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The Excretion of Phosphatase in the Faeces

By N. R. LAWRIE, *From the Biochemical Laboratory, Addenbrooke's Hospital, Cambridge*

(*Received 2 April 1943*)

The mucous membrane of the small intestine is rich in phosphatase which can be liberated into the blood stream or into the intestinal secretion. This accounts for a part of the faecal phosphatase. Estimation of the faecal phosphatase may therefore enable one to study biochemically the activity of the mucous membrane of the small intestine in the intact animal.

The early publications on faecal phosphatase are reviewed by Folley & Kay [1936]. Heymann [1933] found the phosphatase activity to be reduced in rickets, spasmophilia and intestinal infantilism. Armstrong [1936] described the preparation of a strong phosphatase concentrate from dog faeces. Koster's [1938, 1939] most striking finding was the absence of phosphatase from the faeces in some cases of non-tropical sprue. In leukaemia values above the normal range were found. Duckworth & Godden [1941] used faecal phosphatase estimations to measure intestinal secretory activity in rats.

The experiments to be described were performed on the stools of normal adults between 20 and 35 years of age, free from any gross peculiarities of dietetic habit.

EXPERIMENTAL

Estimation of phosphatase

Technique. The stool was weighed, ground with water until homogeneous in a 1 in 400 suspension. Phosphatase was estimated by Bodansky's [1933] method. The increase in 1 hr. in inorganic phosphate, expressed as mg. P/100 g. of untreated or of dried faeces, is referred to as units of phosphatase activity (wet wt.) and (dry wt.) respectively. The former is comparable with the Bodansky unit of phosphatase activity.

Reliability of sampling. Subjects were instructed not to eat foods likely to leave large undigested particles. Grinding of their stools with water gave thorough mixing, and triplicate analyses showed that representative 0·5 g. samples could be obtained.

RESULTS

Solubility of the enzyme

Filtration of the suspensions removed a large and variable proportion of the phosphatase. Thus a stool which contained $2{\cdot}00 \times 10^3$ units (wet wt.) was tested again on four filtrates and the following results obtained: $0{\cdot}11 \times 10^3$, $0{\cdot}13 \times 10^3$, $0{\cdot}13 \times 10^3$, $0{\cdot}15 \times 10^3$ units. Substitution of saline for water did not affect the activity of the suspension.

Activation of the enzyme by Mg

$MgCl_2$ was added to give concentrations of 0·025 and 0·05 % (approx. 0·005 *M*) which was found by Bowers, Outhouse & Forbes [1940] to be optimal for dog faecal phosphatase. The phosphatase in human faeces is not fully activated with regard to Mg (Table 1), but any difference obtained by activation with Mg is negligible in comparison with the individual variations occurring physiologically.

Table 1. *Activation of faecal phosphatase by $MgCl_2$*

Phosphatase activity in units wet wt. $\times 10^{-3}$

Stool no.	Conc. added $MgCl_2$ in digest: Nil	0·025 %	0·050 %	Max. activation % increase
1	3·22	3·58	3·54	11
2	4·28	4·66	4·66	9
3	4·32	4·25	4·62	7
4	15·3	17·3	14·8	13
5	3·52	3·80	3·90	11
6	5·20	—	5·60	8
7	8·6	—	9·6	12
8	9·2	—	10·2	11
9	10·0	—	10·4	4
10	7·34	—	8·00	9

Inhibition of the enzyme by KCN

The results (Table 2) show that this phosphatase is strongly inhibited by 10^{-2} *M* KCN.

Table 2. *Inhibition of faecal phosphatase by KCN*

Stool	Sample	KCN conc. in digest *M*	Phosphatase activity units wet wt.	Inhibition %
A	1	Nil	$2{\cdot}64 \times 10^3$	95
	2	10^{-2}	$0{\cdot}13 \times 10^3$	
	3	Nil	$2{\cdot}74 \times 10^3$	96
	4	10^{-2}	$0{\cdot}11 \times 10^3$	
B	1	Nil	$2{\cdot}96 \times 10^3$	94
	2	10^{-2}	$0{\cdot}11 \times 10^3$	
	3	Nil	$3{\cdot}42 \times 10^3$	97
	4	10^{-2}	$0{\cdot}09 \times 10^3$	

Effect of NaF on activity of the enzyme

Experiments similar to those using KCN (Table 3) show that NaF has no effect upon the phosphatase activity of these preparations.

Table 3. *Effect of NaF upon faecal phosphatase activity*

Stool	Sample	NaF conc. in digest *M*	Phosphatase activity units wet wt.	Activation %
A	1	Nil	$4{\cdot}08 \times 10^3$	1
	2	10^{-2}	$4{\cdot}13 \times 10^3$	
	3	Nil	$4{\cdot}20 \times 10^3$	0
	4	10^{-2}	$4{\cdot}20 \times 10^3$	
B	1	Nil	$3{\cdot}76 \times 10^3$	3
	2	10^{-2}	$3{\cdot}89 \times 10^3$	
	3	Nil	$3{\cdot}75 \times 10^3$	7
	4	10^{-2}	$4{\cdot}02 \times 10^3$	

The phosphatase activity of normal stools

Stools from 50 normal adults were analysed for phosphatase activity and water content. The results are given in Table 4.

Table 4. *Phosphatase activity of normal stools*

	Min.	Max.	Mean of 50
Units wet wt. $\times 10^{-3}$	0·8	36·7	6·0
Units dry wt. $\times 10^{-3}$	1·9	103·0	23·0
Dry matter in stool (%)	15·0	47·0	30·0

DISCUSSION

Cloetens [1939 *a*, *b*; 1940] has presented evidence that the alkaline phosphatase of animal tissues consists of two types, 'alkaline phosphatase I' and 'alkaline phosphatase II'. This classification is based upon activation or inhibition by Mg ions, CN and F. The present results indicate that faecal phosphatase belongs to the subdivision type II of Cloetens, of the group A I of Folley & Kay [1936]. Cloetens found that nearly all the phosphatase of the small intestine of guinea-pigs was of this type.

The most striking feature of the analyses of normal stools is their extreme range of variation; but even the lowest value ($0{\cdot}8 \times 10^3$ units wet wt.) is 200 times the maximum value for normal serum. The values were very unevenly distributed: 36 of them (dry wt. basis) fell below the mean and only 14 above. The maximum value observed by Koster [1939] if converted into comparable units (dry wt.) is 94×10^3, which is close to the present maximum value of 103×10^3. If the mean phosphatase activity of the pooled faeces from six normal rats studied by Duckworth & Godden [1941] is converted into comparable units, a value of $1{\cdot}8 \times 10^3$ (wet wt.) is obtained, which lies within the range of $0{\cdot}8 \times 10^3$ to $36{\cdot}7 \times 10^3$ units (wet wt.) given above for normal human stools.

There is probably a negative correlation between the percentage of dry matter in normal stools and their phosphatase activity. Twenty-four stools containing less than 30 % of dry matter had an average of 33×10^3 units (dry wt.) and 26 stools containing 30 % or more dry matter had 14×10^3. The speed of transit of the stool through the intestine probably influences dry-matter content and phosphatase activity in opposite senses. Koster [1939] states that $MgSO_4$ taken by mouth, or a diet rich in green vegetables, increases the phosphatase activity of the stools. This effect he attributes to activation of the enzyme by Mg ions. Both procedures are, however, likely to increase the speed of transit of the intestinal contents and diminish the percentage of dry matter in the stools.

SUMMARY

1. The phosphatase activity of normal human faeces has been shown to be slightly increased *in vitro* by $0{\cdot}53 \times 10^{-2} M$ $MgCl_2$, uninfluenced by $10^{-2} M$ NaF, and very strongly inhibited by $10^{-2} M$ KCN.

2. The range of variation of the phosphatase activity of 50 normal stools was from $1{\cdot}9 \times 10^3$ to 103×10^3 units (dry wt.) with a mean value of $23{\cdot}0 \times 10^3$ units.

3. There is probably a negative correlation between the phosphatase activity/unit wt. dry matter and the percentage dry matter in normal stools.

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The Amino-acid Composition of Tyrocidine

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(*Received 3 March 1943*)

Tyrocidine hydrochloride, a crystalline antibacterial substance obtained, with gramicidin, from cultures of certain bacteria [Hotchkiss & Dubos, 1941] has been shown [Hotchkiss, 1941; Christensen, Edwards & Piersma, 1941] to be of polypeptide nature. A limited study of the constituent amino-acids has been made by these authors, and by Lipmann, Hotchkiss & Dubos [1941]. Dubos [1942] and Dubos & Hotchkiss [1942] have reviewed its biological properties.

We have studied the products of acid hydrolysis of tyrocidine by partition chromatography [Gordon, Martin & Synge, 1943 *a*, *b*]. The extraction of the acetylation mixture with organic solvents has been rendered almost quantitative, and the use of pelargonin chloride instead of methyl orange has practically eliminated 'leaching' of indicator, facilitating continuous observation of the more slowly moving 'bands'. These changes have extended the quantitative applicability of the method to acetamino-acids, whose bands move more slowly than that of acetylalanine, and although further investigation is necessary before the method can be used for the simultaneous determination of the eight common amino-acids of this group, the present procedure has proved useful with tyrocidine which seems to contain only three of these. Using a variety of solvent systems, we have not yet achieved a satisfactory chromatographic separation of the acetyl derivatives of glutamic and aspartic acid, and have made use of the differential evolution of CO_2 in the presence of ninhydrin from the corresponding free amino-acids [Van Slyke, Dillon, MacFadyen & Hamilton, 1941] for estimating their relative proportions.

From tyrocidine hydrolysates we have isolated and identified as their acetyl derivatives, phenylalanine, leucine, valine, proline, tyrosine, glutamic acid and ornithine, and have obtained further evidence for the presence of tryptophan and aspartic acid [Hotchkiss, 1941]. Alanine, glycine, lysine and hydroxyproline appear to be absent. Christensen *et al.* [1941] claim to have isolated alanine (as its dioxypyridate) but give no details.

All these amino-acids have been obtained predominantly as the *l*-isomers, except phenylalanine, which we find predominantly *d*-, and tryptophan, whose optical configuration was not determined. Many of the specimens isolated were considerably racemized. Our estimate of the total amount of *d*-amino-acids in the hydrolysate is consistent with that of Lipmann *et al.* [1941]. This seems to be the first report of *d*-phenylalanine in nature. The simultaneous incorporation by the same organism of *d*-leucine residues (in gramicidin) and *l*-leucine residues (in tyrocidine) has biological interest.

Ornithine was isolated as its di-acetyl derivative which proved identical with that obtained from *l*-ornithine, described here for the first time. Ornithine has previously been isolated only from hydrolysates of protein or polypeptide material that had been treated in such a way as probably to modify the guanidino group of arginine residues [Kossel & Weiss, 1913]. The negative Sakaguchi reaction of tyrothricin [Hotchkiss & Dubos, 1941] excludes arginine as an immediate precursor, although it must be borne in mind that tyrothricin is obtained from autolysed cultures. The presence of both proline and ornithine in the hydrolysate suggested that citrulline might be a common precursor for both [Wada, 1933], particularly since tyrocidine, like most proteins, gives a positive Fearon [1939] reaction. However, *l*-citrulline, when subjected to the same conditions of acid hydrolysis, acetylation and chromatographic analysis, gave at most 8% of a mol. of proline and 22% of a mol. of ornithine. Since under the same conditions of acid hydrolysis citrulline evolved no CO_2, while urea, another possible product of the reaction postulated by Wada, evolved 42–53% of a mol. of CO_2, and also in view of his inadequate characterization of his product, we disbelieve Wada's statement that proline is the main product formed by boiling citrulline with acid. We believe that ornithine and proline residues exist in the intact molecule of tyrocidine, although the presence of some citrulline is not excluded. Further work on the Fearon reaction of proteins is desirable.

The satisfactory results with the estimation of ornithine given below confirm our view [Gordon *et al.*, 1943*a*] that partition chromatography should provide a satisfactory micro-analytical procedure for the direct determination of lysine.

Quantitative analyses of tyrocidine hydrolysates were carried out and controlled by parallel analyses of a known mixture of the constituent amino-acids in similar quantity and proportion (Table 1). We were struck by the very low and variable recovery

of tryptophan, as in similar control experiments with a different amino-acid mixture [Gordon *et al.*, 1943*b*] higher and less variable recoveries of tryptophan were obtained. Homer [1915] and Onslow [1921, 1924] suggested that other amino-acids might some component not present in our first mixture. Gortner and collaborators (for references cf. Gortner & Norris [1923]) studied the role of tryptophan in humin formation. In our experiments humin formation is negligible although destruction of

Table 1. *Analysis of a known mixture which had been heated with acid under the conditions of hydrolysis employed with tyrocidine*

Known mixture		Analytical results (recovery %)	
Substance present	Amount mg. N	Exp. 1 (batch 'E' gel)	Exp. 2 (batch 'F' gel)
Acetyl-*l*-phenylalanine	0·348	106	101
Acetyl-*d*-leucine	0·290	98·5	105
Acetyl-*l*-valine	0·248	102	102
Acetyl-*l*-tryptophan	0·430	7	42
Acetyl-*l*-proline hydrate	0·158	91	93
Acetyl-*l*-tyrosine	0·167	99	104
Total dicarboxylic acids	0·343	79·5	96
l-Glutamic acid HCl	0·172	45	98 (by diff.)
l-Aspartic acid	0·171	113	94 (by ninhydrin)
Diacetyl-*l*-ornithine	0·493	93·5	96
Ammonium sulphate	0·349	—	—

Table 2. *Analysis of a glutamic acid-tryptophan mixture which had been heated with acid in the presence of, or in the absence of, air*

Known mixture			Analytical results		
			Amino-acids		
Substance present	Amount mg.	Air present during heating ml. at N.T.P.	Found mg.	Recovery %	Carbon dioxide formed mg.
l-Glutamic acid HCl	2·9	0·6	2·46	84	0·14
Acetyl-*l*-tryptophan	6·7		4·05	60	
l-Glutamic acid HCl	2·9	0·0	2·67	92	0·00
Acetyl-*l*-tryptophan	6·7		4·69	70	

Table 3. *Amino-acid analysis of tyrocidine*

(N as % of total N unless otherwise stated)

Preparation analysed ...	Present results A I	A II	B —	Hotchkiss [1941]	Christensen Edwards & Piersma [1941]
mg. N taken for analysis ...	1·56	3·12	2·47		
Phenylalanine	12·2	14·8	12·7	—	12·9
Leucine	8·6	7·4	8·7	—	—
Valine	7·1	8·0	6·5	—	—
Tryptophan	9·5	0·9	1·8	11·7, 15·9*	4·7–6·8
Proline	6·4	6·6	7·1	—	—
Tyrosine	6·4	6·0	6·8	—	7·1
Total dicarboxylic acids	—	12·2	14·1	16·5	15
Glutamic acid (by diff.)	—	6·1	7·2	—	—
Aspartic acid (ninhydrin)	—	6·1	6·9	4·5	—
Ornithine	—	9·3	13·2	—	—
Ammonia	—	—	—	12·0*, 13·3	12·8
Batch of gel used in analysis	E	E	F		

* Determined after brief hydrolysis.

increase the destruction of tryptophan in hot acid, while Holm & Gortner [1920] studied the destruction of tryptophan heated alone with acid. All these workers allowed free access of air during hydrolysis, which is known to influence the destruction of tryptophan. In our experiments air was excluded, and we provisionally attribute the increased destruction to the interaction of tryptophan with tryptophan is almost complete. If this loss is due to interaction with other amino-acids, some destruction of these might be expected, and the results in Table 1 suggest that proline or glutamic acid might be involved. Preliminary experiments described below with tryptophan-glutamic acid mixtures were not decisive, although the evolution of CO_2 when air was present (Table 2) is of interest,

since proteins as usually hydrolysed yield significant quantities of CO_2 [Dunn, 1925]. The hydrolysis of tyrocidine, with exclusion of air, in our hands yielded no CO_2.

The accuracy of the analyses for the other amino-acids appears similar to that previously observed [Gordon *et al.*, 1943*a*]. Some of the minor differences between analyses 1 and 2 (Table 1) are undoubtedly due to differences in the adsorptive properties of the two batches of silica gel employed.

On applying the same analytical procedure to tyrocidine hydrolysates (cf. Table 3) we obtained very variable figures, the extent of the fluctuations being much greater than the experimental error of the method as established by the control experiments. Destruction of tryptophan was with one exception practically complete. We conclude that there are real differences in the composition of different hydrolysates. Incomplete hydrolysis seems an unlikely explanation, since bands corresponding with acetylpeptides should have been seen [Gordon *et al.*, 1943*c*], whereas the appearance of the chromatograms in the analysis of tyrocidine was in no way qualitatively different from that in the control experiments, exactly corresponding 'artefact bands' being observed. Diketopiperazines, however, if present, would not have been observed. We tentatively attribute the discrepancies to the different course which the interaction of tryptophan and other amino-acids may take under the conditions of hydrolysis, when these substances are present in the form of polypeptides rather than as free amino-acids or their simple derivatives.

Further research is required to elucidate the behaviour of tryptophan during acid hydrolysis of proteins. If the existence of interaction with other amino-acids or NH_3 is established, this introduces yet a fresh uncertainty into the deduction of the amino-acid composition of intact molecules from the composition of hydrolysates as usually prepared. With the average protein, of low tryptophan content, the percentage errors introduced in this way would not be as great as with tyrocidine. Nevertheless, they might invalidate stoichiometric calculations for a large molecule.

Whatever general significance for amino-acid analysis our observations may have, the errors encountered have entirely prevented the stoichiometric calculation of a 'minimum molecule' of tyrocidine hydrochloride, since molecules of a composition within the accuracy of our determinations can easily be postulated having two or any larger number of Cl′ groups, while we cannot rigidly exclude a molecule having only one Cl′ group.

Nevertheless, if we combine Hotchkiss's tryptophan and NH_3 figures with those now obtained for other constituents, we can say that 88–105 % of the N of tyrocidine has been identified in its hydrolysate. A reliable estimate of the molecular weight by physical means might throw considerable further light on the data presented here.

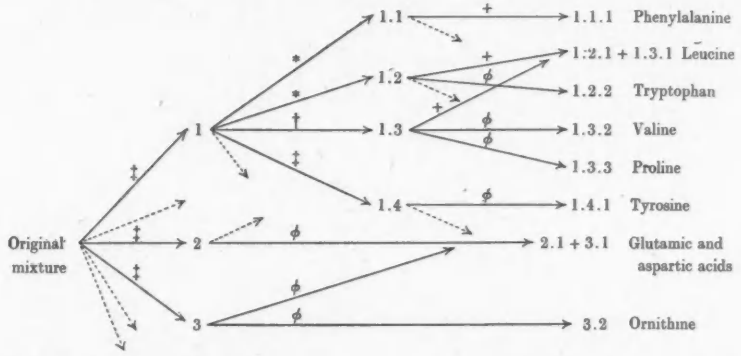
EXPERIMENTAL

General analytical procedure

[cf. Gordon *et al.*, 1943 *a*, *b*]

Unless otherwise stated, preparations were hydrolysed by heating for 24 hr., at the temperature of boiling 6*N* HCl, with 0·8 ml. acetic acid and 3·0 ml. 6*N* HCl in sealed tubes which had been freed from air by boiling the mixture *in vacuo* before sealing [Gordon *et al.*, 1943*b*].

The acetylation and extraction procedure has been modified as follows: 5 ml. 4*N* NaOH were used in place of 10 ml. 2*N* NaOH. After acidification the solution was immediately absorbed in 16 g. silica gel. After thorough



The dotted lines in the diagram correspond to the 'artefact bands' observed during the fractionation.

mixing this was made to a slurry with 17% butanol-$CHCl_3$ (half-saturated with water), and made into a column in the usual way. No indicator was used. The vessels were washed through three times with small quantities of solvent, which were then added separately to the top of the column and allowed to drain in. Finally, fresh solvent to a total of 1 l. was run through the column. This multi-plate 'chromatographic' extraction utilizes solvent much more efficiently than the single-plate separating funnel procedure previously employed.

The scheme of fractionation shown above was employed.

The initial fractionation with 17% butanol-$CHCl_3$ was introduced to ensure complete solution of the material for analysis, which does not occur when 1% butanol-$CHCl_3$ is used first. The silica was saturated with a 0·05% aqueous solution of pelargonin chloride [Gordon *et al.*, 1943*a*].

In calculating N values from the titrations, the following factors were used: ornithine and tryptophan, 2 atoms N; glutamic and aspartic acids, ½ atom N; other fractions 1 atom N per acid equivalent.

Determination of glutamic and aspartic acids

After titration, fraction (2·1 + 3·1) was hydrolysed with 6 *N* HCl for 6 hr. at 100°, evaporated to dryness *in vacuo* and dissolved in water. A suitable portion was taken for ninhydrin-CO_2 determination by the method of Christensen, West & Dimick [1941] with 10 min. heating. Calibration experiments showed that under these conditions glutamic acid evolved 1·02 mol. CO_2, and aspartic acid 1·88 mol. CO_2, and the relative amounts of the two amino-acids present were calculated from this.

CO_2 evolved during hydrolyses

Before analysis of their contents the hydrolysis tubes were connected (still sealed) to the Van Slyke-Neill manometric apparatus through pressure tubing; the tip of the tube was then broken off inside the rubber, the CO_2 transferred to the chamber and determined as described by Van Slyke *et al.* [1941]. Control experiments showed that no CO_2 was evolved by the HCl-acetic acid mixture during 24 hr. heating.

Preparation of tyrocidine hydrochloride

The material studied was obtained from the residues of our preparation of gramicidin from tyrothricin [Gordon *et al.*, 1943*b*]. Preparation A was obtained by three recrystallizations according to Hotchkiss & Dubos [1941]. After the 1st recrystallization, the material had $(\alpha)_D^{17.5-18.5°} = -98°$, after the 2nd, −100°, and after the 3rd, −100·5° (95% ethanol, $c = 1{\cdot}1$–$1{\cdot}3$). Prep. A had N = 14·5% (Kjeldahl, with or without P-HI pretreatment). Prep. B was obtained from A by one further recrystallization.

Characterization of tyrocidine constituents

244 mg. tyrocidine HCl (Prep. A) was hydrolysed and prepared for chromatography as described above, using double the quantity of each reagent. After making the hydrolysate alkaline, NH_3 was removed *in vacuo* before addition of acetic anhydride. The chromatographic fractionation was as above, with columns of suitably enlarged diameter. Pelargonin chloride was used as indicator throughout. Significant quantities of this were eluted by 30% propanol-cyclohexane though not by 17% butanol-$CHCl_3$. In these cases the contaminated fraction, dissolved in a little 17% butanol-$CHCl_3$, was filtered through a layer of 2 g. silica gel saturated with water. By washing with a suitable volume of fresh solvent the acetamino-acid was eluted free from pelargonin.

Acid equivalent weight (A.E.W.) and N (Kjeldahl) determinations were carried out as before [Gordon *et al.*, 1943*b*]; in the former, the first faint persistent pink of phenolphthalein was the end-point. C and H determinations were made by Dr G. Weiler, Oxford. Melting-points are given uncorrected unless otherwise stated. Optical rotations were observed in a 2 dm. tube.

1.1.1. Acetyl-d-phenylalanine. Crystallization from $CHCl_3$ gave a product (M.P. 160°) which on recrystallization yielded 39 mg. having M.P. 164°, which was depressed to 150–158° on admixture of authentic acetyl-*l*-phenylalanine (M.P. 169–171°). $(\alpha)_D^{19.5°} - 37{\cdot}8°$ (ethanol, $c = 1{\cdot}1$). Du Vigneaud & Meyer [1932] record for acetyl-*d*-phenylalanine M.P. 172° (corr.), $(\alpha)_D^{26°} - 51°$ (ethanol). (Found: C, 63·6; H, 6·48; N, 6·53%; A.E.W. 211. Calc. for $C_{11}H_{13}O_3N$: C, 63·7; H, 6·28; N, 6·77%; A.E.W. 207.)

1.2.1 + 1.3.1. Acetyl-l-leucine. Crystallization from $CHCl_3$ yielded 17 mg. having M.P. 180–181°, not depressed on admixture of authentic acetyl-*l*-leucine. Admixture of acetyl-*d*-leucine depressed the M.P. to 160°. $(\alpha)_D^{20.5°} - 19{\cdot}0°$ (ethanol, $c = 0{\cdot}5$). Martin & Synge [1941] record for acetyl-*l*-leucine M.P. 182°, $(\alpha)_D^{24°} - 23{\cdot}5°$ (ethanol, $c = 3{\cdot}3$). (Found: C, 55·7; H, 8·93; N, 7·9%; A.E.W. 175. Calc. for $C_8H_{15}O_3N$: C, 55·5; H, 8·67; N, 8·1%; A.E.W. 173.)

1.2.2. Acetyltryptophan would be expected in this fraction [Gordon *et al.*, 1943*b*]. Crystallization from $CHCl_3$-cyclohexane followed by recrystallization from $CHCl_3$ yielded 4·7 mg. having M.P. 170–190°, not depressed on admixture of acetyl-*l*-tryptophan. $(\alpha)_D^{20.5°}$ slightly positive ($< +3°$) (ethanol, $c = 0{\cdot}15$). After evaporation from ethanol solution N content was 9·1% (P-HI pretreatment), and A.E.W. = 205. (Calc. for $C_{13}H_{14}O_3N_2$: N, 11·4%; A.E.W. 246.) Perhaps this fraction was a mixture of acetylleucine and acetyltryptophan. In two of the three quantitative analyses described below this fraction was also very small; in the other a substantial acid fraction was found.

1.3.2. Acetyl-l-valine. Crystallization from $CHCl_3$ yielded 13 mg. having M.P. 158°, not depressed on admixture of authentic acetyl-*l*-valine. The crystals were contaminated by a water-insoluble gummy material, possibly derived from tryptophan, which proved difficult to eliminate. $(\alpha)_D^{19°} + 2 \pm 1°$ (ethanol, $c = 0{\cdot}35$). Gordon *et al.* [1943*a*] record for acetyl-*l*-valine M.P. 164°, $(\alpha)_D^{19°} + 4{\cdot}0°$ (ethanol, $c = 2$). (Found: C, 54·8; H, 8·22; N, 8·0%; A.E.W. 176. Calc. for $C_7H_{13}O_3N$: C, 52·8; H, 8·17; N, 8·8%; A.E.W. 159.)

1.3.3. Acetyl-l-proline (monohydrate). Crystallization from wet ethylacetate yielded 23 mg. having (air-dry) M.P. 76–78°, not depressed on admixture of authentic acetyl-*l*-proline hydrate. $(\alpha)_D^{19°} - 112{\cdot}5°$ (H_2O, $c = 0{\cdot}7$) (rotation calc. for anhydrous compound). Martin & Synge [1941] record M.P. 78°, $(\alpha)_D^{22°} - 114{\cdot}0°$ (H_2O, $c = 4{\cdot}2$). Analyses were on the air-dry hydrate. (Found: C, 47·2; H, 7·26; N, 7·7%; A.E.W. 184. Calc. for $C_7H_{11}O_3N.H_2O$: C, 48·0; H, 7·43; N, 8·0%; A.E.W. 175.)

1.4.1. Acetyl-l-tyrosine. Treatment with ethanol-light petroleum eliminated much gummy material, after which

were slowly deposited 17 mg. crystals (slightly contaminated by gum) having M.P. 148°, not depressed on admixture of authentic acetyl-*l*-tyrosine. $(\alpha)_D^{19.5°}+41°$ (H_2O, $c=0.4$). Du Vigneaud & Meyer [1932] record M.P. 152–154° (corr.), $(\alpha)_D^{26°}+47.5°$ (H_2O, $c=2$). (Found: C, 57.6; H, 5.53; N, 5.9%; A.E.W. 252. Calc. for $C_{11}H_{13}O_4N$: C, 59.3; H, 5.83; N, 6.3%; A.E.W. 223.)

2.1+3.1. Dicarboxylic amino-acids. A preliminary determination on a portion of this fraction (*R* (chromatogram band rate) = 0.2 (approx.) on both 17% butanol-$CHCl_3$ and 30% propanol-cyclohexane columns [Gordon *et al.*, 1943*a*]) showed exactly 2 acid equiv./atom N, suggesting the presence of dicarboxylic acids only. Since attempts at crystallization led only to mixed products, the material was refractionated on a 17% butanol-$CHCl_3$ chromatogram, and the front and back portions of the resulting broad band were collected separately. Crystallization of the former material from ethanol-$CHCl_3$ yielded 6 mg. having M.P. 190°, not depressed on admixture of authentic *acetyl-l-glutamic acid*. Bergmann & Zervas [1928] record M.P. 199° (corr.). (Found: C, 44.9; H, 5.71; N, 8.4%; A.E.W. 91. Calc. for $C_7H_{11}O_5N$: C, 44.4; H, 5.82; N, 7.4%; A.E.W. 94.5.) The material available did not suffice for optimum analytical accuracy.

In a second analysis, the corresponding fraction from the hydrolysate of 210 mg. of Prep. A was hydrolysed (6 hr. in 6*N* HCl at 100°), and evaporated to small volume. The dicarboxylic acids were separated in the usual way. Saturation with HCl gas at 0° gave slow deposition of glutamic acid HCl crystals (16 mg.), having $(\alpha)_D^{18.3°}+13°$ (H_2O, $c=0.3$) (rotation calc. for glutamic acid). Wood [1914] records for *l*-glutamic acid in water + 1 mol. HCl $(\alpha)_D^{25°}+27.9°$. (Found: N, 7.3%; A.E.W. 93. Calc. for $C_5H_9O_4N$. HCl: N, 7.6%; A.E.W. 92.)

The mother liquors from the above, after most of the HCl had been removed *in vacuo*, were treated with copper acetate in 2 ml. water. The crystalline Cu salt deposited was filtered off and decomposed in the usual way with H_2S, yielding material (5.2 mg.) which did not readily crystallize from water, having N content (7%) lower than the theoretical for aspartic acid. After treatment with excess HCl followed by evaporation to dryness, the material had $(\alpha)_D^{18°}+13°$ (H_2O, $c=0.2$) (rotation calc. for dry wt. before HCl treatment). Wood [1914] records for *l*-aspartic acid in H_2O + 0.94 mol. HCl $(\alpha)_D^{25°}+18.4°$. A ninhydrin-CO_2 determination showed the same CO_2/N ratio as aspartic acid (see above). From this identity, the insolubility of the Cu salt, the —COOH/N ratio, and the fact that the rate and appearance of the dicarboxylic acid chromatogram band from tyrocidine hydrolysates could be closely simulated by mixtures of acetylglutamic and acetylaspartic acids, we conclude that the fraction under consideration was an impure specimen of aspartic acid, and that tyrocidine hydrolysates contain *l*-glutamic and *l*-aspartic acids, the former considerably racemized.

3.2. NN′-diacetyl-l-ornithine. Crystallization from ethylacetate and from ethanol-ether successively yielded 25 mg. needles having M.P. 156°, not depressed on admixture of authentic diacetyl-*l*-ornithine (see below). $(\alpha)_D^{16°}+4°$ (ethanol, $c=0.4$). (Found: C, 50.6; H, 7.77; N, 12.3%; A.E.W. 224.) On 17% butanol-$CHCl_3$ and 30% propanol-cyclohexane chromatograms the band-rate *R* was approx. 0.1, and the material was chromatographically indistinguishable from authentic diacetyl-*l*-ornithine on a butanol-$CHCl_3$ column, while readily separable from diacetyl-*dl*-lysine [cf. Gordon *et al.*, 1943*a*].

Acetylation of l-ornithine

l-Ornithine HCl prepared from *l*-arginine HCl by the action of liver arginase [Hunter, 1939] and having $(\alpha)_D^{18°}+10.8°$ (H_2O, $c=5$) was subjected to acetylation and extraction as described above, and yielded material similar in appearance and crystallization properties to the product from tyrocidine, having M.P. 156° (constant on recrystallization), $(\alpha)_D^{15°}+6.3°$ (ethanol, $c=0.8$). (Found: C, 49.9; H, 7.68; N, 12.7%; A.E.W. 218. $C_9H_{16}O_4N_2$ requires: C, 50.0; H, 7.40; N, 13.0%; A.E.W. 216.)

Quantitative analyses of tyrocidine hydrolysates, etc.

Three analyses were performed, according to the general scheme given above, on 10–20 mg. tyrocidine HCl with 3 g. silica gel in 1 cm. tubes for the butanol-$CHCl_3$, and 2 g. for the other fractionations. Methyl orange was substituted for pelargonin in those columns where the development was begun with 1% butanol-$CHCl_3$ or 5% propanol-cyclohexane, because of its superior sensitivity.

Control analyses on a known mixture after heating in acid are shown in Table 1. Similar experiments on simple mixtures of glutamic acid and acetyltryptophan were carried out in the presence and absence of air. In the former analysis air was readmitted to the 20 ml. tube to a pressure of 4 cm. Hg after the tube had been completely evacuated. The analytical procedure was simplified by omitting the propanol-cyclohexane chromatograms. Table 2 indicates the slightly higher recoveries found in the absence of air and gives the CO_2 evolution measured as described above. No CO_2 was evolved by tyrocidine or by the control mixture or by 4.05 and 6.1 mg. *l*-citrulline (Hofmann La Roche—kindly provided by Prof. H. A. Krebs) when heated in acid under the same oxygen-free conditions. 1.65 and 2.5 mg. of urea similarly treated yielded 0.565 and 0.98 mg. CO_2 respectively. After 4 mg. citrulline had been treated as above the resulting material was analysed in the same way as the glutamic acid-tryptophan mixtures, yielding at most 8% of a mol. of acids having $R>0.5$ in 17% butanol-$CHCl_3$ (which would include acetylproline), and 22% of a mol. of diacetylornithine. These substances were not identified; the figures are based on titration of bands having corresponding rates. (For recovery of proline and ornithine from known mixture cf. Table 1.)

Table 3 shows the results (uncorrected) of our analyses of tyrocidine together with the figures obtained by other workers.

SUMMARY

1. Technical modifications have extended quantitative analysis by partition chromatography to a further group of amino-acids.

2. From an acid hydrolysate of tyrocidine, phenylalanine, leucine, proline, valine, tyrosine, ornithine and glutamic acid have been isolated by partition chromatography, and characterized as their acetyl derivatives. Additional evidence has been obtained for the occurrence of tryptophan and

aspartic acid. This appears to be the first isolation of ornithine by acid hydrolysis of an intact protein or polypeptide.

3. The phenylalanine isolated had mainly the *d*- configuration; the other amino-acids were mainly *l*-.

4. 88–105 % of the N of tyrocidine has now been accounted for, but the variability of the analytical figures has not permitted stoichiometric calculations. The implications of this variability, which exceeds the analytical errors, are discussed.

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The Structure of the Galactose-phosphate Present in the Liver During Galactose Assimilation

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In this paper further support is provided for the view [Kosterlitz, 1937; 1938; 1939*b*] that in the galactose-phosphate present in the liver during galactose assimilation the phosphate group is attached to carbon atom 1. It has not yet been possible to separate this ester from the accompanying glycerophosphate, and from small quantities of glucose-1-phosphate and an unidentified ester. But the facts that reducing hexosemonophosphates can be removed by alkaline hydrolysis, and that Ba glycerophosphate is very resistant to acid hydrolysis and has a specific rotation of 0°, have made possible the approximate determination of the rate of acid hydrolysis of the galactose-phosphate and of its specific rotation. Since the ester is non-reducing, resistant to alkaline hydrolysis and very labile to acid hydrolysis, it is concluded that the first carbon atom is substituted. The velocity constant (k) of hydrolysis of the natural ester in 0·25 N HCl at 25° is $0{\cdot}91 \times 10^{-3}$, while that of synthetic galactose-1-phosphoric acid is $0{\cdot}89 \times 10^{-3}$. Natural Ba galactose-1-phosphate has $[\alpha]_{5461} + 113°$ while the synthetic ester has $[\alpha]_{5461} + 109{\cdot}5°$. These values strongly suggest that the natural and synthetic esters are identical. It is of interest to note that Wolfrom & Pletcher [1941] and Wolfrom, Pletcher & Brown [1942] have succeeded in finally proving that the glucose-1-phosphate of Cori, Colowick & Cori [1937] has the structure of phosphoryl α-mono-*d*-glucopyranoside. Since the principle of the synthesis of galactose-1-phosphate is analogous to that devised by Cori *et al.* [1937] for glucose-1-phosphate, the findings of Wolfrom *et al.* [1942] give further support to the view expressed on other grounds [Kosterlitz, 1939*a*] that the synthetic galactose-1-phosphate is phosphoryl α-mono-*d*-galactopyranoside.

EXPERIMENTAL

The general methods were the same as those described in previous papers [Kosterlitz, 1937; 1939*a*]. The Ba salts of the phosphoric esters were obtained

from the livers of 38 rabbits which had been fed with galactose. The method of isolation has been described previously [Kosterlitz, 1937]. No nitrogenous substances were present in the ester mixture. The yield of Ba salts was 1·75 g. from 2·41 kg. liver.

Analysis of Ba salts of the ester mixture. The substance contained 6·87% total P and 1·88% acid-labile P (=27·4% of the total P). The initial reduction was 9·1% 'glucose'; 12 min. hydrolysis in 0·09 *N* H_2SO_4 at 100° increased the reduction by 8·9% 'glucose' or 11·7% 'galactose'. The fermentation analysis gave the following results: change after fermentation with *Saccharomyces ludwigii* (*S. ludwigii*) = +2·8% 'glucose'; change after fermentation with galactose-adapted *Saccharomyces cerevisiae* (*S. cerevisiae*) Frohberg = −8·9% 'glucose'; residual reduction: 3·0% 'glucose'. The mixed Ba salts, therefore, contained 11·7% galactose as ester or 25·7% anhydrous Ba galactose-1-phosphate. Since Ba glycerophosphate was found to be more easily soluble in aqueous ethanol than Ba galactose-phosphate, a separation of the two compounds was attempted by fractional precipitation with aqueous ethanol. This attempt, however, was not very successful [Kosterlitz, 1938].

Acid and alkaline hydrolysis of the ester mixture. A sample of a composition slightly different from that described in the preceding paragraph was used for this particular experiment. 17·96 mg. Ba salt were dissolved in 15 ml. H_2O containing 0·87 ml. *N* H_2SO_4 and the precipitated $BaSO_4$ removed by centrifuging. The initial reduction was 7·76% 'glucose' and the total P 7·02%. The acid-labile P, determined by 3 min. hydrolysis in 0·09 *N* H_2SO_4 at 100°, was 1·84%; the alkali-labile P, determined by hydrolysis in 0·2 *N* NaOH at 100°, was 1·29% after 20 min. and 1·33% after 30 min. hydrolysis. The acid-labile P constituted 26·2% and the alkali-labile P 18·7% of the total P so that 55% of the total P was resistant to hydrolysis. Galactose-1-phosphate and glucose-1-phosphate were the main sources of the acid-labile P, and reducing hexosephosphates and possibly pentosephosphates those of the alkali-labile P.

Removal of reducing phosphate esters by hydrolysis in 0·2 N NaOH. 534 mg., containing 11·7% galactose (=62·5 mg.) as phosphate, were dissolved in 47·4 ml. H_2O and 2·58 ml. *N* H_2SO_4. The precipitated $BaSO_4$ was removed by centrifuging and washed with 5 ml. H_2O. To the total of 55 ml. were added 2·58 + 13·75 ml. *N* NaOH (final concentration = 0·193 *N*). The hydrolysis was allowed to proceed at 100° for 30 min., during which time the solution turned deeply yellow, due to the action of the alkali on the reducing sugars. After cooling and addition of 16·33 ml. *N* HCl, $Ba(OH)_2$ was added until the *p*H was 8·4. The precipitated Ba phosphate was centrifuged off and washed. To the supernatant fluid, ethanol was added to give a concentration of 60%. After 2 hr. standing at 0°, the material was centrifuged, the supernatant fluid decanted, and the precipitate successively extracted with 20, 20, 10, 10 and 15 ml. H_2O. The residue was dissolved in a minimum of 0·25 *N* HCl, the solution brought back to *p*H 8·4 by means of $Ba(OH)_2$, and then centrifuged. The supernatant fluid was added to the aqueous extracts, followed by ethanol to give a concentration of 60%. The extraction of the centrifuged precipitate was repeated twice and the final precipitate dried *in vacuo* over P_2O_5. Yield: 293 mg. This material together with 23 mg. from a previous experiment (containing 3·0 mg. galactose as phosphate) was dissolved in 26 ml. H_2O, the insoluble material separated and washed by centrifuging. To the clear solution (32 ml.) an equal quantity of ethanol was added; after standing at 0° for 2 hr., the Ba salts were centrifuged off, washed with 50 and 80% ethanol and dried *in vacuo* over $CaCl_2$. Yield: 295 mg. The slightly turbid solution of this product in 10 ml. H_2O was centrifuged, and an equal quantity of ethanol was added to the supernatant fluid. After standing at 0° for 2 hr., the precipitate was centrifuged off and then successively extracted with 15, 10 and 15 ml. 10% ethanol. Ethanol was added to the supernatant fluid to give a concentration of 50%. After 2 hr. standing at 0°, the precipitate was centrifuged off, washed with 80% ethanol and dried *in vacuo* over $CaCl_2$. The Ba salts were now completely soluble in H_2O. Yield: 228 mg. containing 16·3% = 37·2 mg. galactose as phosphate. The crude material contained 62·5 mg. (+3·0 mg.) galactose. Yield: 57%.

Analysis of the Ba salts of the ester mixture freed from reducing phosphates. The substance contained 7·46% total P and 2·77% acid-labile P (=37·2% of total P). $[\alpha]_{5461}^{20°}$ = +34·4 (*c* = 1·476; *l* = 2). The initial reduction was negligible (0·45% 'glucose') and 3 min. hydrolysis in 0·09 *N* H_2SO_4 at 100° increased the reduction by 12·4% 'glucose' or 16·3% 'galactose'. The fermentation analysis (Table 1) showed that most of this increase was due to galactose:

Table 1. *Analysis of saccharide phosphate mixture by fermentation*

	Unhydrolysed esters	Hydrolysed esters
Initial reduction	0·38% 'glucose'	12·9% 'glucose'
Change after fermentation with *S. ludwigii*	+10·9% 'glucose'*	− 1·53% 'glucose'
Change after fermentation with galactose-adapted *S. cerevisiae* Frohberg	−10·5% 'glucose'	−10·2% 'glucose'
Residual reduction	=13·8% 'galactose'	=13·3% 'galactose'
	0·72% 'glucose'	1·15% 'glucose'

* This increase in reduction was caused by phosphatatic hydrolysis of galactose-1-phosphate [Kosterlitz, 1937; 1939*a*].

From these results, the following conclusions may be drawn: (1) alkaline hydrolysis practically abolished the initial reduction which was caused by reducing hexosephosphates and possibly pentosephosphates; (2) the greatest part but not all of the reducing substances produced by acid hydrolysis consisted of galactose (13·5%). Besides galactose, 1·53% glucose was liberated, and 0·93% expressed as glucose of a non-fermentable reducing substance. Thus, the mixture of Ba salts contained as anhydrous salts: 29·6% Ba galactose-1-phosphate, 3·4% Ba glucose-1-phosphate, and 2·0% unidentified acid-labile ester (calculated on basis of hexosephosphate). This made a total of 35% anhydrous Ba salt or 39·8% Ba salt containing $3H_2O$.

These results show up a fact which so far has escaped attention, namely, that during galactose assimilation, glucose-1-phosphate is present in the

liver besides galactose-1-phosphate. In experiments on rats, Kosterlitz & Ritchie [1943*b*] could demonstrate an increase in glucose-1-phosphate in the livers after administration of galactose as well as of glucose. Nothing can so far be said about the unknown acid-labile ester.

Hydrolysis in 0·09 N H_2SO_4 at 100° of the ester mixture freed from reducing phosphates

(Concentration of acid-labile P = 0·525 m.-molar)

Time min.	Increase in reduction mg. 'glucose'/ mg. Ba salt	Increase in P mg./mg. Ba salt
2	0·125	0·0272
3	0·125	0·0277
10	0·126	0·0277
180	0·130	0·0318

The liberation of reducing substances and of P was practically complete after 2–3 min. hydrolysis; under the conditions of this experiment, galactose-1-phosphate is completely hydrolysed in 2 min. and glucose-1-phosphate in 5 min. [Kosterlitz & Ritchie, 1943*a*]. Between 10 and 180 min. there was an insignificant increase in reduction, while there was still a liberation of inorganic P; this would suggest the presence of a non-reducing difficultly hydrolysable ester (or esters) such as glycerophosphoric acid.

Isolation of the difficultly hydrolysable esters by acid hydrolysis of the ester mixture freed from reducing phosphates. 96·5 mg. Ba salts were dissolved in 29·5 ml. H_2O and 3·49 ml. *N* H_2SO_4 (= free esters in 0·09 *N* H_2SO_4). This solution was hydrolysed at 100° for 10 min. After addition of $Ba(OH)_2$ (*p*H 8·4), the precipitate was centrifuged off and 2 vol. of ethanol added to the supernatant fluid. The precipitate was dissolved in the minimum amount of 0·25 *N* HCl, the solution warmed in order to precipitate colloidal $BaSO_4$, and the $BaSO_4$ centrifuged off. $Ba(OH)_2$ was added to the supernatant fluid (*p*H 8·4), the precipitate of inorganic P centrifuged off, and 2 vol. of ethanol added. The $BaSO_4$ was warmed with 0·25 *N* HCl and the HCl extract treated as described above. The combined aqueous alcoholic mixtures were allowed to stand for 2 hr. at 0°. After centrifuging, the precipitate was exhaustively extracted with H_2O and the centrifuged extracts treated with 2 vol. ethanol. After standing at 0° for 2 hr., the precipitate was centrifuged and the process of extraction repeated once with H_2O and a second time with 10% aqueous ethanol. The final precipitate was dried *in vacuo* over $CaCl_2$. Yield: 39 mg. Expected: 60% of 96·5 mg. = 58 mg.; yield: 67%.

Analysis of the Ba salt of the difficultly hydrolysable ester. Total P = 7·48%; inorganic P = 0; acid-labile P = 0. Initial reduction = 0·58% 'glucose'; acid-labile reduction = 0·3% 'glucose'. $[\alpha]_{5461}^{17.5°} = -3.4°$ ($c = 0.778$; $l = 2$).

Fermentation analysis: initial reduction = 0·61% 'glucose'; change after fermentation with *S. ludwigii* = +0·38% 'glucose'; change after fermentation with galactose-adapted *S. cerevisiae* = −0·57% 'glucose'; residual reduction = 0·42%.

This ester was practically non-reducing; it was not further analysed but was probably composed mainly of Ba α-glycerophosphate (+ $3H_2O$ = 8·6% P) which is present in liver in considerable quantities [Fiske & Subbarow, 1929]. However, the low P value and the slight laevo-rotation indicated that other unknown esters were also present.

Calculation of the specific rotation of natural Ba galactose-1-phosphate. The Ba salts of the ester mixture containing 29·6% anhydrous galactose-1-phosphate had $[\alpha]_{5461}$ +34·4°. By deducting −2·0° for the 60% believed to be present of the Ba salt of the difficultly hydrolysable ester, and +3·0° for 3·4% anhydrous Ba glucose-1-phosphate, a value of +33·4° was obtained, which corresponded to 29·6% anhydrous Ba galactose-1-phosphate. Thus, natural anhydrous Ba galactose-1-phosphate has approximately $[\alpha]_{5461} = +113°$, while for the anhydrous Ba salt of the synthetic ester $[\alpha]_{5461} = +109.5°$. No allowance could be made for the 2% unidentified acid-labile ester.

Hydrolysis of the acid-labile fraction of the ester mixture, freed from reducing phosphates in 0·25 N HCl at 25°. 28·19 mg. Ba salt were dissolved in a total of 10 ml. with addition of 1 drop sat. K_2SO_4 solution and 1·365 ml. of 0·1 *N* H_2SO_4, in order to liberate the free acid. After centrifuging off the $BaSO_4$, 9 ml. 0·5 *N* HCl were added to 9 ml. of the supernatant fluid. The concentration of acid-labile P was 1·17 m.-molar.

Hydrolysis in 0·25 N HCl at 25°

Time min.	Increase in reduction (% of total acid-labile reduction)	$k \times 10^3$	Inorganic P formed (% of total acid-labile P)	$k \times 10^3$
180	29·6	0·847	29·8	0·854
255	38·3	0·764	38·6	0·775
330	45·8	0·751	46·0	0·744
	Average	0·787		0·791

Calculation of the k of hydrolysis of natural galactose-1-phosphoric acid by 0·25 N HCl at 25°. The acid-labile fraction of the ester mixture contained 84·6% galactose-1-phosphoric acid, 9·7% glucose-1-phosphoric acid, and 5·7% unidentified ester. The *k* of the acid-labile fraction was 0.79×10^{-3}. The *k* of glucose-1-phosphoric acid (0.2×10^{-3}) was calculated from the known *k* values of the glucose-1-phosphoric and galactose-1-phosphoric acids at 25°, 37° and 50° [Cori *et al.*, 1937; Kosterlitz, 1939*a*; Kosterlitz & Ritchie, 1943*a*]. No allowance could be made for the 5·7% unidentified ester. By deducting 0.02×10^{-3} for the 9·7% glucose-1-phosphate present in the acid-labile fractions a value of 0.77×10^{-3} was obtained, which corresponded to 84·6% galactose-1-phosphoric acid. Thus, the *k* of natural galactose-1-phosphoric acid is approximately 0.91×10^{-3}, while the *k* of the synthetic ester is 0.89×10^{-3}.

SUMMARY

1. The mixture of phosphoric esters isolated from the livers of rabbits assimilating galactose was subjected to alkaline hydrolysis in order to remove reducing hexosephosphates.

2. The product obtained in this way contained 29·6% anhydrous Ba galactose-1-phosphate, 3·4% anhydrous Ba glucose-1-phosphate and 2% Ba salt of unidentified acid-labile ester; the remainder consisted of a difficultly hydrolysable non-reducing ester. The Ba salts of the ester mixture freed from

reducing phosphates had $[\alpha]_{5461} + 34{\cdot}4°$ and the Ba salt of the difficultly hydrolysable ester $[\alpha]_{5461} - 3{\cdot}4°$. From these values it was calculated that the natural anhydrous Ba galactose-1-phosphate has $[\alpha]_{5461} + 113°$, while the synthetic product has $[\alpha]_{5461} + 109{\cdot}5°$.

3. The velocity constant (k) of hydrolysis of the acid-labile fraction of the ester mixture, freed from reducing phosphates, in $0{\cdot}25N$ HCl at 25° was $0{\cdot}79 \times 10^{-3}$. From this value the k of the natural galactose-1-phosphoric acid was calculated to be $0{\cdot}91 \times 10^{-3}$, while the k of the synthetic product is $0{\cdot}89 \times 10^{-3}$.

4. These results strongly suggest that the natural and synthetic galactose-1-phosphoric acid are identical. The ester probably is an α-pyranoside as is the natural glucose-1-phosphoric acid.

An expenses grant by the Medical Research Council which partly defrayed the cost of this investigation is gratefully acknowledged.

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The Apparent Dissociation Constants of Galactose-1-Phosphoric Acid

By H. W. KOSTERLITZ, *From the Physiology Department, Marischal College, Aberdeen*

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Meyerhof & Suranyi [1926], Meyerhof & Lohmann [1927], and Cori, Colowick & Cori [1937] found that the dissociation constants (pK' values) of hexose-diphosphoric, hexose-6-monophosphoric, fructose-6-phosphoric, and glucose-1-phosphoric acids were smaller than those of orthophosphoric acid. In the present paper α(?)-galactose-1-phosphoric acid is shown to have dissociation constants similar to those of the other hexosemonophosphates. The knowledge of the acidic properties of galactose-1-phosphoric acid is necessary for the assessment of the changes in pH which may be brought about by the phosphorylation of galactose.

EXPERIMENTAL

The pH values were measured by means of a Cambridge pH meter with dip glass electrode (portable pattern). A buffer consisting of equal parts of Na acetate and acetic acid ($0{\cdot}1N$) and having pH 4·62 was used for standardizing the glass electrode. The galactose-1-phosphoric acid was prepared by treating the Ba salt (from the K_2 salt [Kosterlitz, 1939]) with the exact quantity of H_2SO_4. The concentration of galactose-1-phosphoric acid in the final solution was determined by estimation of phosphorus. 10 ml. of $0{\cdot}1015M$ (Exp. 1) and $0{\cdot}1038M$ (Exp. 2) galactose-1-phosphoric acid were titrated with CO_2-free $0{\cdot}0999N$ NaOH (temp. = 18–20°). For comparison, 10 ml. of $0{\cdot}1001M$ H_3PO_4 were similarly titrated. The results were plotted after correction for the deviations from 0·1 molarity

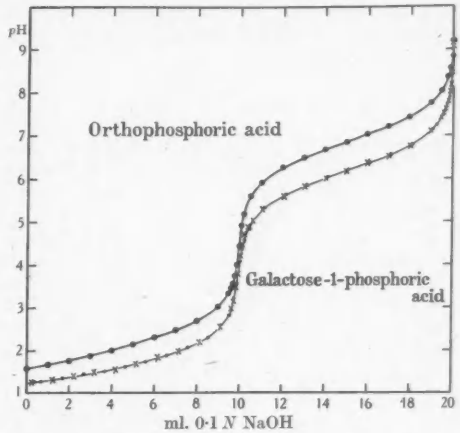

Fig. 1. 10 ml. $0{\cdot}1M$ orthophosphoric acid •—• 10 ml. $0{\cdot}1M$ galactose-1-phosphoric acid: Exp. 1 ·—·; Exp. 2. ×—×.

(Fig. 1). The dissociation constants were calculated from 3 to 4 values each; pK'_1 by means of

$$K'_a = \frac{[H]^+ (B + [H]^+)}{C - (B + [H]^+)}$$

and pK'_2 by means of Henderson-Hasselbalch's equation

$$pK'_a = p\mathrm{H} - \log \frac{B}{C-B}$$

[Van Slyke, 1922]. In these equations B stands for molar concentration of total alkali added and C for molar concentration of galactose-1-phosphate in the titration mixture at any given time.

The values found for orthophosphoric acid, pK'_1 1·95 and pK'_2 6·83, agree well with those recorded in the literature [Van Slyke, 1922; Meyerhof & Suranyi, 1926; Cori *et al.*, 1937]. Galactose-1-phosphoric acid has pK'_1 1·00 and pK'_2 6·17 while hexose-6-phosphoric acid (Robison ester) has pK'_1 0·94 and pK'_2 6·11, fructose-6-phosphoric acid pK'_1 0·97 and pK'_2 6·11 [Meyerhof & Lohmann, 1927] and glucose-1-phosphoric acid pK'_1 1·10 and pK'_2 6·13 [Cori *et al.*, 1937]. There is no significant difference between the biologically important pK'_2 values of galactose-1-phosphoric acid and those of the other hexosemonophosphates.

Since Meyerhof & Lohmann [1927] found that the pK_2 values of fructose-6-phosphoric and hexose-6-phosphoric (Robison) acids were decreased by about 0·14 by the addition of 0·1 M neutral salt solution, 10 ml. 0·1 M K_2 galactose-1-phosphate solution were titrated with N HCl. pK'_2 determined in this way was 6·06, as compared with 6·17, the value obtained by titrating the ester with NaOH.

SUMMARY

α(?)-Galactose-1-phosphoric acid, like other hexose-monophosphates, is a stronger acid than is orthophosphoric acid. α(?)-Galactose-1-phosphoric acid has pK'_1 1·00 and pK'_2 6·17, while orthophosphoric acid has pK'_1 1·95 and pK'_2 6·83.

An expenses grant by the Medical Research Council which partly defrayed the cost of this investigation is gratefully acknowledged.

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The Fermentation of Galactose and Galactose-1-phosphate

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Galactose-1-phosphate is present in the livers of rabbits and rats during galactose assimilation [Kosterlitz, 1937; Kosterlitz & Ritchie, 1943*b*]. No information as to the fate of this ester has so far been obtained except that it is more resistant to the phosphatase of liver than is glucose-1-phosphate [Cori, Colowick & Cori, 1938; Kosterlitz & Ritchie, 1941]. Since the carbohydrate metabolism of yeast cells is strikingly similar to that of mammalian tissue, it was decided to examine the fermentation of galactose and galactose-1-phosphate and compare the results with those obtained in the fermentation of glucose and glucose-1-phosphate.

The ability of some yeasts to ferment galactose is due to the formation of 'galactozymase' during adaptation. This process takes place in aqueous solutions of galactose and can occur without cell multiplication [Stephenson & Yudkin, 1936; for the older literature see that paper, and Harden, 1932]. Yeast juice [Harden & Norris, 1910; Grant, 1935], dried yeast and maceration extract [Abderhalden, 1925] prepared from adapted yeast, will ferment galactose. In the products of fermentation Nilsson [1930] found, besides Harden-Young ester, a monophosphate with a specific rotation higher than that of Robison ester. No data as to the reducing power of this ester and the nature of its constituent hexose were given. Grant [1935] confirmed the presence of 1:6-fructose diphosphate, and found trehalose-monophosphate and Robison ester. Using the methylphenylhydrazine test, he could not find any evidence for the accumulation of a galactose phosphoric ester. He further could demonstrate that galactose-6-phosphate was not fermented by galactose-adapted yeast.

EXPERIMENTAL

Materials and methods

Maceration extracts. These were prepared in the usual manner from a pure strain of *Saccharomyces cerevisiae* (*S. cerevisiae*) Frohberg, which had been propagated in this laboratory since 1935, when it was generously supplied by

Dr H. B. Hutchinson of the Distillers Company, Ltd. It has been subcultured in broth containing 0·8% dried 'Difco' yeast extract and 3% galactose. For the preparation of dried yeast, the yeast was grown in aerated broth in lots of 1½–3 l. at 25° for 48 hr.

Galactose. In order to remove glucose, a 10% solution of commercial galactose was incubated with 2% *Saccharomyces ludwigii* (*S. ludwigii*) at 28° for 48 hr.; after centrifuging off the yeast and concentrating the solution under reduced pressure, the galactose was crystallized several times from 70% ethanol. It had $[\alpha]_{5461}^{17°} + 95{\cdot}0°$.

Glucose-1-phosphate. The K_2-salt was prepared according to Hanes [1940].

Galactose-1-phosphate. The K_2-salt was prepared by the method given in a previous paper [Kosterlitz, 1939*a*]. Since the K_2-salts of the esters are alkaline, HCl was added to obtain the desired *p*H. The quantities of acid required were read from electrometric titration curves [Kosterlitz, 1943].

CO_2 production. This was followed in Warburg manometers shaken in a bath at 25°. The gaseous phase was N_2; any O_2 present in the commercial nitrogen was absorbed by passing through two gas-washing bottles containing a solution of 16 g. $Na_2S_2O_4.2H_2O$ and 2 g. Na-anthraquinone-2-sulphonate in 100 ml. 13% NaOH [Fieser, 1924] and through a third bottle containing 13% NaOH.

Estimation of glucose-1-phosphate and galactose-1-phosphate. The esters were determined in trichloroacetic acid filtrates by the method described by Kosterlitz & Ritchie [1943*a*].

RESULTS

The results of three typical experiments are given in Fig. 1*a–c*. In all experiments, whether with hexoses or esters, typical curves of CO_2 production were obtained, showing an induction period, a period of rapid fermentation (period of esterification), and finally, a period of slow fermentation (period of hexosediphosphate fermentation). Meyerhof & Lohmann [1927] found that only the naturally occurring hexosemonophosphates, namely, Neuberg and Robison esters, gave such curves, while synthetic esters which were not intermediaries in alcoholic fermentation produced CO_2 at a slow and uniform rate.

In the first experiment (Fig. 1*a*), notwithstanding the addition of a small quantity of hexosediphosphate to the fermentation mixture, the CO_2 curves of the free hexoses showed a fairly long induction period and an autocatalytic shape. The maximum rates of CO_2 production were 80 μl./5 min. with glucose, and 62 μl./5 min. with galactose. On the other hand, the curves obtained with glucose-1-phosphate and galactose-1-phosphate were practically identical. Their maximum rates were 45–50 μl. CO_2/5 min. It is to be noted that, since in these experiments inorganic P was present in excess, the shape of the CO_2 curves was wholly conditioned by the substrate. Thus, during the rapid period of fermentation, glucose produced 575 μl. CO_2 and galactose 500 μl. CO_2 instead of the expected 1090 μl., or about 50%, while the esters produced 300 μl. CO_2 or 27·5% of the theoretical value. This means that while the hexoses obeyed the Harden-Young equation the esters did not. These results are in good agreement with those of Meyerhof & Lohmann [1927] who, investigating the action of maceration extract on Neuberg and Robison esters, found a CO_2 production of 25% of the calculated value, and with those of Harden & Robison [Harden, 1932] who found that during fermentation with zymin, only 20–25% of the added Robison ester was fermented at the enhanced rate. The lower CO_2 production by the monophosphates can be explained by the facts that, compared with the hexoses, they can accept only one-half the quantity of phosphate and that, according to Warburg & Christian [1939], the partial dephosphorylation of 1:3-diphosphoglyceric acid to 3-phosphoglyceric acid by the hexokinase-adenosine diphosphate-hexose system is a limiting factor in alcoholic fermentation by maceration extract.

Addition of catalytic quantities of acetaldehyde and hexosediphosphate (Fig. 1*b*) almost abolished the induction period in the fermentation of the free hexoses, with a simultaneous increase of the maximum rates of CO_2 production. In the case of glucose the maximum rate rose from 80 to 160 μl./5 min., and in the case of galactose from 62 to 75 μl./5 min. Although the addition of acetaldehyde caused a marked difference between the fermentation rates of free glucose and free galactose, it scarcely influenced the fermentation of either of the 1-esters.

When acetaldehyde alone was added to the fermentation mixture, the difference between the fermentations of glucose and galactose became rather more marked than in the experiment with addition of acetaldehyde and hexosediphosphate. There was a slight difference between the rates of CO_2 production in the case of the 1-esters in favour of glucose-1-phosphate (Fig. 1*c*).

If the yeast was dried slowly in the manner suggested by Lipmann [1938], the maceration extract obtained from it was four times as active as were extracts from yeasts dried in the ordinary way. In the presence of acetaldehyde, the maximum rates of CO_2 evolution/5 min. were as follows: glucose 690 μl., galactose 270 μl., glucose-1-phosphate and galactose-1-phosphate 200 μl. With glucose-1-phosphate the maximum fermentation rate was attained sooner than with galactose-1-phosphate, otherwise there was no difference between the fermentations of the two esters.

The experiments described so far indicate that galactose-1-phosphate plays the same role in galactose fermentation as do glucose-1-phosphate and glucose-6-phosphate in glucose fermentation. Grant [1935] found that Robison ester and 1:6-fructose diphosphate accumulate during fermentation of galactose by adapted yeast preparations, and that

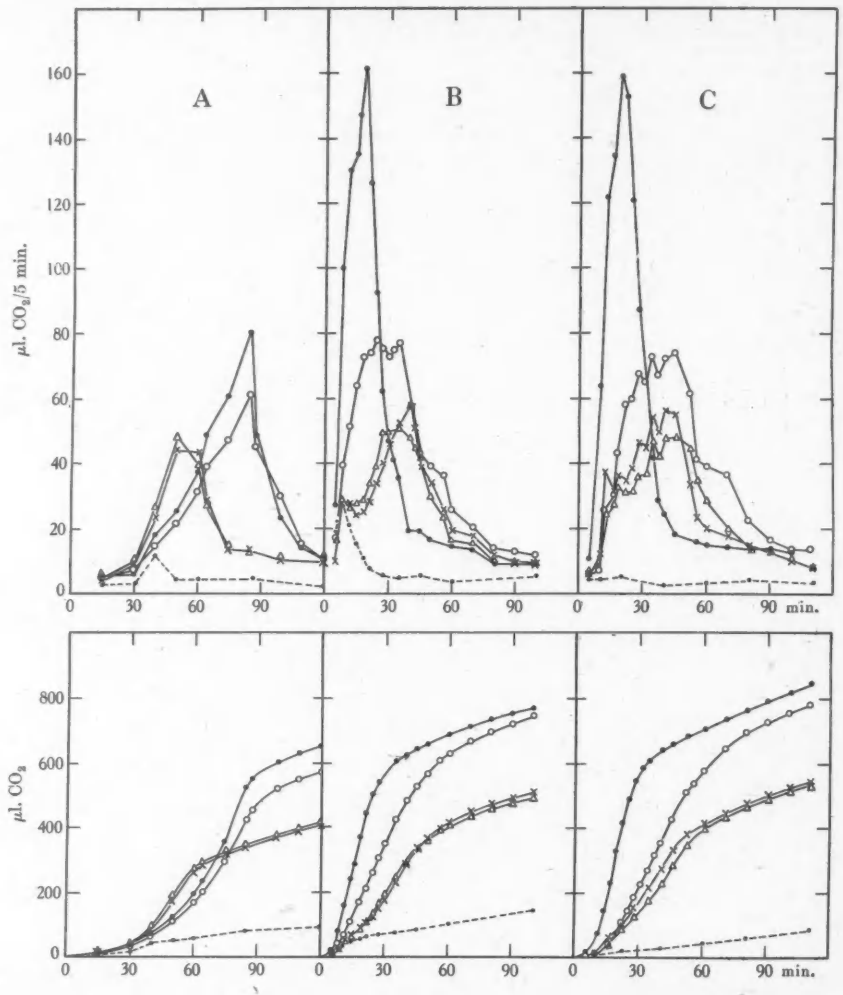

Fig. 1. Maceration extract from *Saccharomyces cerevisiae* Frohberg grown on galactose broth. 1 part dried yeast and 4 parts water were incubated at 35° for 2 hr. The extract was obtained by centrifuging at 4000 r.p.m. 1 ml. contained 2·55 mg. inorganic P; *p*H 5·8.

Main chamber: 1 ml. extract.

Side-bulb: (*a*) 0·05 ml. K_2-hexosediphosphate (0·115 mg. P), and either 0·25 ml. hexose solution (=4·37 mg. hexose=1090 μl. CO_2) in 0·097 *M* phosphate buffer of *p*H 6·5, or 0·25 ml. ester solution (=4·37 mg. hexose) in 0·0267 *N* HCl (*p*H 6·5).

(*b*) 0·05 ml. containing Mg-hexosediphosphate (0·07 mg. P) and 1 mg. acetaldehyde, and either 0·2 ml. hexose solution (=4·5 mg. hexose=1120 μl. CO_2) in 0·12 *M* phosphate buffer of *p*H 6·5, or 0·2 ml. ester solution (=4·5 mg. hexose) in 0·0338 *N* HCl (*p*H 6·5).

(*c*) as in (*b*), but hexosediphosphate was omitted.

····· Autofermentation; •—• glucose; o—o galactose; ×—× glucose-1-phosphate; △—△ galactose-1-phosphate.

galactose-6-phosphate is not an intermediary in galactose fermentation. The question, therefore, arises of how galactose-1-phosphate is converted to Robison ester: Is galactose-1-phosphate transformed to glucose-1-phosphate, which in turn is acted upon by phosphoglucomutase and isomerase to form Robison ester? Preliminary experiments with dialysed maceration extracts were not conclusive on account of the presence of phosphoglucomutase in such extracts. To give an example: 0·75 ml. extract, dialysed for 2 hr. + 0·5 ml. 0·08 *M* veronal acetate buffer of *p*H 6 + 0·25 ml. 0·06 *M* glucose-1-phosphate or galactose-1-phosphate adjusted to *p*H 6 were incubated at 25° for 30 min. In the case of glucose-1-phosphate 83 % of the ester was converted to difficultly hydrolysable ester (Robison), 14 % was accounted for by inorganic P, while 3 % remained unaltered. In the case of galactose-1-phosphate only 27 % was converted to difficultly hydrolysable ester, 11 % was accounted for by inorganic P, while 62 % remained unaltered. The structure of the difficultly hydrolysable ester formed from galactose-1-phosphate has not yet been identified but it is likely to be Robison ester, since galactose-6-phosphate can be ruled out. No glucose-1-phosphate was present in the reaction mixture of the experiment with galactose-1-phosphate, a finding which had to be expected in view of the presence of highly active phosphoglucomutase. Unfortunately, phosphoglucomutase cannot be inhibited specifically. Attempts to reduce the activity of phosphoglucomutase by prolonged dialysis were accompanied by great losses in the activity of the galactose-1-phosphate-converting enzyme; the activity could not be restored by Mg^{++} ions which, in extracts dialysed for 2 hr., accelerated the disappearance of galactose-1-phosphate. Fractionation with $(NH_4)_2SO_4$ has so far been unsuccessful; both the galactose-1-phosphate-converting enzyme and phosphoglucomutase were absent from the proteins precipitated by either 50 or 70 % saturation.

Notwithstanding the great differences between the fermentation rates of the non-phosphorylated hexoses, the fermentation curves of the 1-esters are so similar to each other as to suggest the existence of an enzymatic equilibrium between the esters. However, in order to obtain further information on the mechanism which causes the conversion of galactose-1-phosphate to Robison ester, the attempts to isolate the enzyme, or at least to separate it from phosphoglucomutase, will have to be taken up again on a scale larger than is possible at present.

From the facts described in this paper, a tentative hypothesis of galactose fermentation may be formulated as follows: During the adaptation of *S. cerevisiae* Frohberg to ferment galactose at least two new enzymes are formed. Enzyme (1) phosphorylates galactose at C_1, probably by a system similar to the hexokinase-adenylpyrophosphate systems which phosphorylate glucose and fructose at C_6 and not at C_1 [Colowick & Kalckar, 1941]. Enzyme (2) converts galactose-1-phosphate to Robison ester, probably by way of glucose-1-phosphate, which in turn is acted upon by phosphoglucomutase or isomerase. The fermentation rate of galactose is lower than that of glucose; the rates of reaction (1) or (2) or of both constitute the limiting factors. During the process of adaptation, yeast may be fully adapted to ferment galactose-1-phosphate and, at the same time, be incompletely adapted to ferment non-phosphorylated galactose; when this is the case, galactose will be fermented more slowly than the 1-phosphate. This phenomenon, which was observed in two out of five samples of dried yeast [Kosterlitz, 1939*b*; 1942], supports the view that two new enzymes are formed during adaptation. Non-adapted yeast ferments neither galactose nor galactose-1-phosphate.

SUMMARY

1. With maceration extract, both glucose-1-phosphate and galactose-1-phosphate showed the characteristics of natural hexosemonophosphates, namely, the three periods of induction, rapid fermentation, and slow fermentation. While the non-phosphorylated hexoses produced about 50 % of the calculated quantity of CO_2 during the period of rapid fermentation, the esters produced only 25–30 % during the same period. The fermentation rates of the esters were so similar as to suggest an enzymatic equilibrium between them. So far it has not been possible to supply direct experimental proof for this suggestion.

2. A tentative hypothesis of galactose fermentation is formulated: it is assumed that during the adaptation of *Saccharomyces cerevisiae* Frohberg to ferment galactose, two new enzymes are formed, one which phosphorylates galactose at C_1 and another which converts galactose-1-phosphate to Robison ester, probably by way of glucose-1-phosphate.

An expenses grant by the Medical Research Council which partly defrayed the cost of this investigation is gratefully acknowledged.

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The Oxidation of Ascorbic Acid by *o*-Dinitrobenzene, and the Detection of Dehydroascorbic Acid

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During a survey of the natural sources of ascorbic acid, three reactions have been observed that may be of analytical value: (1) the rapid reduction of *o*-dinitrobenzene by ascorbic acid to a substance giving a violet pigment in alkaline solution, (2) the formation of a green pigment when dehydroascorbic acid is boiled alone in aqueous solution at *p*H 3·5–4·5, and (3) the spontaneous formation of a red pigment when dehydroascorbic acid is kept in alkaline solutions out of contact with air.

(1) *Reaction of ascorbic acid with* o*-dinitrobenzene*

(*a*) *Experimental.* When 2–5 ml. of a dilute (0·02 %) aqueous solution of ascorbic acid are treated in the cold with 5 drops of a saturated aqueous solution of *o*-dinitrobenzene, and then made alkaline by addition of 5 drops 20 % NaOH, a violet colour rapidly develops, and is stable for several hours. Under these conditions the colour is not given by dehydroascorbic acid, cysteine, glutathione, uric acid or creatinine. Fructose gives a slow positive reaction in the cold, but only after 10–15 min., when the solution contains at least 0·2 % of this sugar. The other reducing sugars may require 2 hr. or longer. Proteins, simple alcohols, aldehydes, ketones, and the commoner hydroxy-, keto-, and amino-acids do not give the test. The test loses its selectivity if the reaction mixture is heated, and a positive result is then given by all the reducing sugars and by uric acid. Thus, by controlling temperature and concentration of alkali, it becomes possible to detect ascorbic acid in presence of other compounds of biological importance. On acidification, the violet pigment changes to yellow, and can be extracted by chloroform or by peroxide-free ether. Addition of alkali removes the pigment from the organic solvent, and regenerates the violet colour. By this means, small amounts of ascorbic acid can be detected in solutions the colour of which obscures the test.

(*b*) *History of the reagent.* Lipschitz [1920] reported that living tissues, such as muscle and yeast, contain a catalytic system capable of reducing '*m*-dinitrobenzene' to a yellow pigment, at *p*H 7·4, under anaerobic conditions. Addition of alkali converted the yellow into violet. The test was developed colorimetrically, and it was later found to depend on the presence of *o*-dinitrobenzene as a contaminant in the *m*-isomer [Lipschitz & Osterroth, 1924]. Apparently unaware of this, Ekkert [1934] claimed that reducing sugars gave a violet colour when heated in alkaline solution with *m*-dinitrobenzene, but v. Szecseny-Nagy [1935] showed that this reaction was due also to contamination by the *o*-isomer, and that pure *m*-dinitrobenzene gave no colour. This was confirmed by Truhaut [1937].

(*c*) *Mechanism of the test.* From an inspection of the formulae of the reactants, the test seems to depend on the presence of the enediol system, —C(OH)=C(OH)—, which occurs in ascorbic acid, though, of course, the reagent is not specific for this system, since powerful inorganic reducing agents such as hydrosulphite give a similar response.

Aldoses and ketoses react only when conditions bring about enolization. Kept in alkaline solution at room temperature for some hours they give an immediate violet on addition of the reagent, which

thus may be used to demonstrate the relative rate of transformation. Polyhydroxy compounds incapable of enolization, such as glycerol, mannitol, sorbitol, mucic and tartaric acids, do not react. Pyrogallol, when dissolved in oxygen-free water, and protected from atmospheric oxidation by a layer of toluene, gives an immediate reaction, as also does 1:3-dihydroxyacetone, and glucoreductone.

The pigment formed by the reduction of *o*-dinitrobenzene has been investigated by Bamberger & Hübner [1903], and by Meisenheimer [1903]. Using hydroxylamine as the reducing agent, the end-product was identified as *o*-nitro-nitrosobenzene, and isolated as the Na salt. Working under less drastic conditions, Patzig & Patzig [1906] were able to recover *o*-dinitrobenzene from the pigment after acidification and atmospheric oxidation.

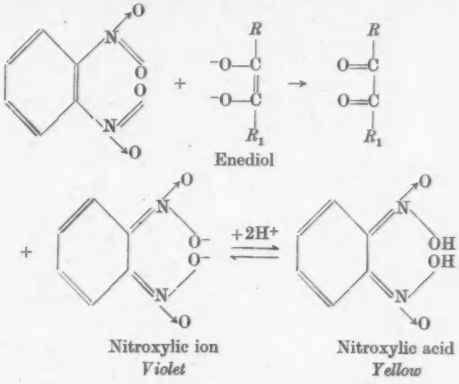
The reaction with ascorbic acid has been found to proceed on similar lines. When equimolecular proportions of ascorbic acid and *o*-dinitrobenzene interact in *N*/10 NaOH, the primary product is the violet-coloured ion of the *o*-nitroxylic acid, which on acidification is converted into the yellow form of the free acid, soluble in ether.

After recrystallization from ethanol, the acid separates out in wisps of very fine yellow needles which turn violet on exposure to NH_3. Melting-point determinations were unsatisfactory, as the acid readily dehydrates to *o*-nitro-nitrosobenzene, which is recognizable by the fact that it gives no violet colour on addition of alkalis.

The low solubility of *o*-dinitrobenzene in water (about 0·03 % at room temperature) restricts the yield of the pigment, while if the ascorbic acid be in excess at the start of the reaction the reduction proceeds beyond the nitroxylic stage.

(*d*) *Preparation of the pigment.* 100 mg. ascorbic acid (Roche or B.D.H.) were dissolved in 50 ml. Cu-free distilled water in which were suspended 100 ml. finely powdered *o*-dinitrobenzene. 1 ml. 20 % NaOH was added drop by drop, the mixture being shaken continuously. A deep violet colour appeared immediately, and became more intense as the dinitrobenzene slowly dissolved. After 12–24 hr. at room temperature, all the ascorbic acid had been oxidized, as was shown by withdrawing samples, discharging the violet colour by acetic acid, and then titrating with standard 2:6-dichlorophenolindophenol. The mixture was then filtered free from undissolved dinitrobenzene, and treated with CO_2 until the colour became orange-yellow. It was then concentrated *in vacuo*, acidity being maintained by passing in CO_2 whenever the violet colour reappeared. At the end of 2–3 days, yellow crystals of *o*-nitro-nitrosobenzene (M.P. 87–90°) began to separate out in small clusters on the surface. The yields were only about 10 % of the theoretical, the bulk of the original dinitrobenzene having undergone further reduction to a mixture of at least three different products, which separated out in a yellow crystalline crop when the solution was concentrated to a syrup.

In terms of current formulation, the primary reaction is:



(2) *Pigment formation from dehydroascorbic acid alone*

(*a*) *At* p*H 4.* 5 ml. of a dilute (0·005–0·5 %) aqueous solution of dehydroascorbic acid, adjusted to *p*H 3·5–4·5 by means of an acetate-acetic acid buffer, and gently boiled for a couple of minutes, develops a stable grass-green colour. The colour is not given by the reduced form of ascorbic acid, unless boiling is prolonged sufficiently to allow of atmospheric oxidation. Other reducing agents, such as cysteine, glutathione, the monosaccharides, uric acid and creatinine, do not give the test. Colour formation is not dependent on the presence of acetate ions, but can be observed in mixtures suitably buffered by phosphate or $(NH_4)_2SO_4$ and H_2SO_4. The green colour is stable for several hours, but may turn brown if the solution is too concentrated, the acidity insufficient, or the boiling greatly prolonged. The solution may be diluted with water for colorimetric comparison. Multivalent ions, especially Al^{+++}, Ca^{++}, Zn^{++}, and Pb^{++}, intensify the colour and change it to yellow, but accelerate the subsequent bleaching by atmospheric oxidation.

The reaction can be demonstrated by buffering 3 ml. of a 0·1 % aqueous solution of ascorbic acid by addition of about 1 g. of solid Na acetate and 1 ml. of glacial acetic acid. The mixture is then carefully oxidized by addition of 1 % iodine drop by drop, any excess of iodine being bleached by addition of a particle of thiourea or a drop of ascorbic acid solution, as it tends to destroy the pigment. After boiling the mixture for 1 min., the green colour appears and reaches a maximum within 5 min. The pigment can be extracted directly by amyl alcohol, or by ethanol or acetone after saturation of the mixture with $(NH_4)_2SO_4$. Addition of alkali converts the green into a red pigment rapidly bleached by exposure to air. Formation of the green pigment can also be observed when dehydroascorbic acid is kept in contact with concentrated HCl at room temperature, or warmed gently, but the test is best obtained by boiling at *p*H 4.

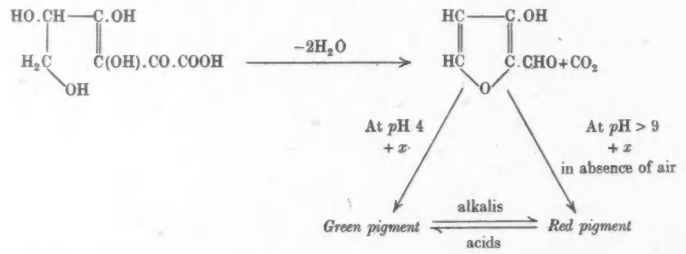
The reaction is not obviously affected by proteins, amino-acids, sugars, or the urinary solutes; and as none of the common biological compounds examined gives a similar response, the test can be used to show the presence of dehydroascorbic acid in natural sources, such as the white inner rind of citrus fruits. It can be applied to coloured solutions after acidification by acetic acid and agitation with 'norite' charcoal, which also oxidizes any ascorbic acid present. Although the intensity of the green pigment is not great, small amounts may be detected by extraction into an amyl alcohol layer.

(*b*) *At* p*H* > 9. When 5 ml. of a 0·1 % aqueous solution of dehydroascorbic acid, preferably in freshly boiled water, are made strongly alkaline by addition of about 1 ml. 20 % NaOH, and protected from atmospheric oxidation by a layer of toluene, a bright yellow-green colour develops within 30 min. at room temperature, and in the course of some hours slowly changes into a deep carmine red. The red pigment is rapidly bleached by exposure to air during the stage of its formation, but is stable for upwards of a week if protected from oxidation, unless the mixture be too strongly alkaline, when it changes to brown and tends to precipitate. The colour reaction is not given by reduced ascorbic acid, reducing sugars, simple ketones or other enolizable compounds. Colour formation may be hastened by gentle warming, but the pigment is then less stable. While the green precursor of the red pigment closely resembles the green pigment obtained from dehydroascorbic acid at *p*H 4, it is not identical, since it is bleached by acidification, whereas acidification of the red pigment changes it into a green indistinguishable from the *p*H 4 pigment. The red pigment in neutral solution yields a series of lakes, especially with Al and Zn, and also can be separated by adsorption on $MgCO_3$ suspensions.

(3) *Mechanism of the tests*

Pending actual isolation of the pigments, which has not yet been accomplished, the following suggestions are advanced. Since freshly prepared dehydroascorbic acid is colourless, it presumably does not contain the chromogenic 2:3-diketone system, =CH—CO—CO—*R*, but exists as the enol form, =C=C(OH)—CO—*R*. Hence, it was first assumed that the *p*H 4 green pigment was merely the true diketone form of the oxidized vitamin. This is disproved by the fact that the pigment does not yield an osazone with phenylhydrazine, and cannot be reconverted into recoverable ascorbic acid by reduction. During the process of boiling, CO_2 is evolved, which suggests that the —CO—COOH end of the chain is being decarboxylated, as would happen if a furfural derivative were being formed. That a furfural is obtained by the action of acids on dehydroascorbic acid can be demonstrated by warming some of the solution with an equal volume of concentrated HCl and a few mg. of pyrogallol, when a deep purple colour develops. Reduced ascorbic acid gives this reaction only on prolonged heating and aeration. The *p*H 4 green pigment does not give the pyrogallol reaction, nor can furfural be obtained by distillation of the mixture, which indicates that furfural formation precedes the formation of the green pigment, and enters into its construction.

Furfural can also be detected in the alkaline colour reaction by addition of acetone, which combines with it to form difurfurylacetone, stable to atmospheric oxidation and yielding a bright carmine on acidification. This can be demonstrated only in the early stages of the test.



The fact that ascorbic acid is quantitatively converted into furfural by boiling with HCl is well known [Cox, Hirst & Reynolds, 1932; Roe, 1934], while the development of a yellow colour in alkaline solutions of the vitamin has been noted [Herbert, Hirst, Percival, Reynolds & Smith, 1933], but neither the acid green pigment nor the red alkali pigment appears to have attracted attention, although the latter may be concerned in the reaction described by Kruger [1906], in which milk made strongly alkaline and kept at room temperature for several days often develops a red colour.

SUMMARY

1. A colour reaction between ascorbic acid and *o*-dinitrobenzene is described.

2. Solutions of dehydroascorbic acid spontaneously form pigments under certain conditions.

3. The specificity of these colour reactions is described, and their mechanism discussed.

We are indebted to Messrs Roche Products Ltd., for the gift of ascorbic acid, and to the Medical Research Council of Eire for the grant to one of us (E. K.) to enable this work to be done.

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Studies in Detoxication

13. THE BIOSYNTHESIS OF AMINOPHENYL- AND SULPHONAMIDOAMINOPHENYL-GLUCURONIDES IN THE RABBIT AND THEIR ACTION ON HAEMOGLOBIN *IN VITRO*

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(*Received 12 February 1943*)

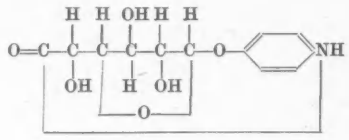
Sieberg [1914] fed 'cupferron' (ammonium salt of nitroso-β-phenylhydroxylamine) to dogs and isolated from their urine a crystalline compound, M.P. 179–180°, which he claimed to be the *lactam* form (I) of *p*-aminophenylglucuronide. He isolated the same compound from urine after the administration of β-phenylhydroxylamine. When this substance was neutralized with Na_2CO_3 and the solution kept for a day, it was able to convert a solution of haemoglobin to methaemoglobin. It should be noted that the β-glycosidic link present in this compound is stable to dilute Na_2CO_3, so that in this case formation of methaemoglobin could not be ascribed to hydrolysis of the glucuronide to *p*-aminophenol, which is a methaemoglobin-former *in vitro* and *in vivo* [Bernheim, Bernheim & Michel, 1937]. From this it would appear that the 'detoxicated' form of *p*-aminophenol could still exert a toxic effect on blood. Therefore, it was important to confirm this observation of Sieberg and to extend it to the study of the properties of the aminophenyl- and sulphonamidoaminophenylglucuronides, none of which has been prepared or described hitherto.

Aminophenylglucuronides

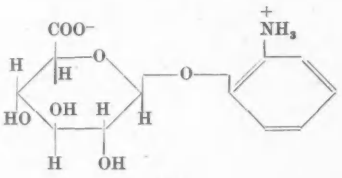
Aminophenylglucuronides are produced in the animal body in response to the administration of the aminophenols themselves, or of certain aromatic amino compounds which are oxidized or converted in some way to aminophenols. When aromatic amino compounds are oxidized *in vivo* to aminophenols, *o*- or *p*-phenols only are produced and in no case studied has *m*-aminophenol been detected.

o-Aminophenol is produced when dimethylaniline or its oxide [Horn, 1936] or acetanilide [Jaffe & Hilbert, 1888] is fed to dogs, or when salvarsan is injected into humans [Sieberg, 1916]. It is excreted in conjugation both with sulphuric and glucuronic acids, and in the rabbit some 26–29% (dose, 0·25 g./kg.) is excreted as an ethereal sulphate [Williams, 1938]. In the present work *o*-aminophenylglucuronide has been isolated from the urine of rabbits fed with *o*-aminophenol. The yield of the crystalline glucuronide was equivalent to 25·5% of the phenol fed and since no free aminophenol was excreted, the remainder, if excreted, must be in

other conjugated forms. *o-Aminophenyl-β-d-glucuronide* ($[\alpha]_D - 76{\cdot}1°$ in $N/10$ HCl) is a white crystalline solid which does not melt, is insoluble in cold water and ordinary organic solvents, but soluble in acid, alkali and boiling water. Its properties indicate that it is probably best formulated as an internal salt (II):



I



II

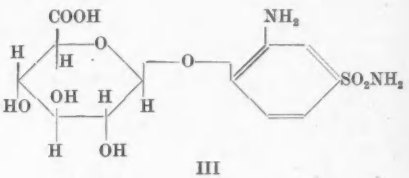
m-*Aminophenylglucuronide* ($[\alpha]_D = -94°$ in HCl) was prepared similarly. About 22 % of the *m*-aminophenol fed was isolated as the glucuronide. Williams [1938] had previously shown that 31–36 % (dose, 0·25 g./kg.) is excreted by the rabbit as an ethereal sulphate. The glucuronide is very similar in properties to the *o*-compound and is likewise probably an internal salt (cf. II).

The fate of *p*-aminophenol in the body has received more attention than that of its isomerides, because it is formed *in vivo* from aniline, acetanilide and a number of related compounds, and its formation is claimed to be responsible for the cyanosis, etc., produced by drugs containing these compounds. It has been suggested that the antipyretic action of such substances as acetanilide and *p*-ethoxy-acetanilide (phenacetin) may be due to slow formation *in vivo* of *p*-aminophenol [see Bernheim *et al.* 1937]. *p*-Aminophenol is excreted as an ethereal sulphate in the dog [Baumann & Herter, 1877–8] and in man [Mörner, 1889]. Williams [1938] showed the 19–20 % of *p*-aminophenol (dose, 0·25 g./kg.) was excreted by the rabbit as an ethereal sulphate. On feeding *p*-aminophenol to rabbits the glucuronide isolated was hydrated *p-aminophenyl-β-d-glucuronide* ($C_{12}H_{15}NO_7$, H_2O) and not Sieberg's compound (I). Like Sieberg's compound, however, *p*-aminophenylglucuronide neutralized with Na_2CO_3 converts haemoglobin to methaemoglobin *in vitro*.

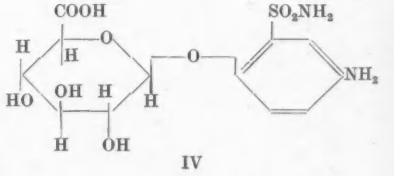
p-Aminophenylglucuronide was similar in many properties to its *o*- and *m*-isomers, but differed in that it showed a definite melting-point and contained water of crystallization. The amount of the glucuronide isolated was about 8 % of the phenol fed, and unlike the *o*- and *m*-compounds, free *p*-aminophenol was present in the urine.

Sulphonamidoaminophenylglucuronides

It was suggested [Thorpe, Williams & Shelswell, 1941] that 3-hydroxy-4-aminobenzenesulphonamide might be an oxidation product of sulphanilamide *in vivo*. Shelswell & Williams [1940] showed that sulphanilamide increased the ethereal sulphate output of rabbits, thus indicating the probable formation of a phenol from sulphanilamide in the animal [cf. also Sammons, Shelswell & Williams, 1941; Williams, 1941]. Such an aminophenolsulphonamide could be excreted as a conjugated glucuronide (cf. sulphapyridine which increases the glucuronic acid output in rats [Scudi & Robinson, 1941]). Aminophenolsulphonamides were therefore fed and an attempt was made to isolate the resulting sulphonamidoaminophenylglucuronides. 4-Hydroxy-3-amino-, 6-hydroxy-3-amino- [Williams, 1942] and 3-hydroxy-4-aminobenzenesulphonamide [Thorpe & Williams, 1941] were fed and crystalline glucuronides were isolated in the first two cases, but in the third, although a glucuronide was formed it was not obtained crystalline but as an amorphous Ba salt which has not yet been purified. The crystalline compounds were *4-sulphonamido-2-aminophenylglucuronide* (III), M.P. 150° and $[\alpha]_D - 67{\cdot}7°$, and *2-sulphonamido-4-aminophenylglucuronide* (IV) which does not melt and shows $[\alpha]_D - 76{\cdot}9°$. Their properties were very similar to those of the aminophenylglucuronides and when neutralized with Na_2CO_3 they converted haemoglobin to methaemoglobin *in vitro*:



III



IV

The action of the above glucuronides on haemoglobin in vitro

It was found that the *o*- and *p*-aminophenylglucuronides when neutralized with Na_2CO_3 converted

a solution of oxyhaemoglobin* to methaemoglobin, the change being observed spectroscopically. The tests were carried out for a period up to 6 hr., but as a rule the methaemoglobin-formers produced their effect in 2–3 hr. No change in the spectrum of the control solution occurred in this time. Fairley [1940] found that a concentrated solution of human haemoglobin incubated at 37° began to form methaemoglobin spontaneously within 20 hr., but the present experiments were not long enough for this. The results of the present experiments are shown in Tables 1 and 2.

The *o*-compound appeared to be several times more effective than the *para*-. A slight formation of methaemoglobin was also observed in the case of the *m*-compound, although *m*-aminophenol itself was quite ineffective in this respect. The neutralized solutions of the glucuronides were quite stable since no free aminophenols were obtained from them on continuous extraction with ether, although if present they would readily have been extracted. If red corpuscles contained β-glucuronidase, hydrolysis of these glucuronides might have occurred. Whilst extracts of ox and dog kidney, liver and spleen hydrolysed conjugated glucuronides, the latter were unaffected when incubated with ox or dog blood [Sera, 1915]. Oshima [1934] and Fishman [1940] make no reference to the occurrence of β-glucuronidase in blood. It is unlikely, therefore, that the aminophenylglucuronides would undergo spontaneous or enzymic hydrolysis under the conditions of the present experiments. It is, however, significant that the most effective methaemoglobin-formers are the *o*- and *p*-compounds, i.e. derivatives of *o*- and *p*-aminophenols which themselves can form iminoquinones. The formation of iminoquinones does not appear possible with the aminophenylglucuronides.

The sulphonamidoaminophenylglucuronides (III and IV) were also methaemoglobin-formers, as were also the corresponding aminophenolsulphonamides and particularly the aminophenolsulphonic acids. The *o*-, *m*- and *p*-aminobenzenesulphonamides were ineffective *in vitro*, although it is well known that sulphanilamide forms methaemoglobin *in vivo*, probably due to its conversion to an aminophenolsulphonamide.

The present findings, therefore, confirm and extend Sieberg's original observation. *o*- and *p*-aminophenylglucuronides and the sulphonamido-*o*- and -*p*-aminophenylglucuronides convert haemoglobin to methaemoglobin *in vitro*. Discussion of the significance of these results, and their translation to *in vivo* conditions, however, must await further investigation.

* Lysed human red cells or solutions of Hb washed free from plasma were used in these experiments in order to avoid a possible complication due to methaemalbumin (pseudo-methaemoglobin) formation which may occur when whole human blood is used [see Fairley, 1938; 1940; 1941].

EXPERIMENTAL

Isolation of the aminophenylglucuronides

o-Aminophenylglucuronide. Six rabbits (2·5 kg.) were each given 1 g. of *o*-aminophenol with water by stomach tube. In about 1 hr. the excreted urine showed the presence of conjugated aminophenol and increased amounts of glucuronic acid. In 3·5 hr. 300 ml. of urine collected which gave an intense naphthoresorcinol reaction, a red colour with nitrous acid and dimethyl-α-naphthylamine (diazo reaction), but did not reduce ammoniacal $AgNO_3$. The urine was acidified with a few drops of glacial acetic acid and treated with saturated normal lead acetate until no further precipitate was formed. The filtrate was neutralized with NH_3 and treated with excess saturated basic lead acetate solution. The precipitate was centrifuged, washed twice on the centrifuge with water, and the lead salt suspended in water and decomposed with H_2S. The filtrate from the PbS was concentrated *in vacuo* at 45–50°, when the *o-aminophenylglucuronide* began to separate as a white crystalline solid, and finally taken to dryness. The white residue was shaken with ethanol and the whole filtered. The glucuronide was then washed with ethanol and ether (yield 3·3 g.). A second portion of urine (210 ml.), collected during the 2 hr. following the first, yielded 0·7 g. of the glucuronide. The total yield for 5 hr. was 4 g. or 25·5% of the aminophenol fed. Later specimens of urine contained very little of the glucuronide.

The glucuronide was recrystallized from a large volume of hot water and formed cigar-shaped platelets which blackened without melting on heating to 300°. It was insoluble in cold water, ethanol, ether and most organic solvents, sparingly soluble in hot water, but soluble in acid and alkali. It showed $[\alpha]_D - 76{\cdot}1°$ ($c = 1$ in $N/10$ HCl). Found: C, 50·7; H, 5·35; N, 4·9%. $C_{12}H_{15}O_7N$ requires C, 50·5; H, 5·3; N, 4·9%.

m-*Aminophenylglucuronide.* Six rabbits were each given 1 g. of *m*-aminophenol with water by stomach tube. The urine was worked up in two portions as described above for the *ortho* compound. The first portion (375 ml.), collected during 3 hr., yielded 2·4 g. of m-*aminophenylglucuronide*; the second, collected during the next 4 hr., gave 1·03 g.: total yield 3·45 g. or 21·9% of the aminophenol fed. The urine gave a bluish red diazo reaction, an intense naphthoresorcinol reaction and was non-reducing.

The glucuronide recrystallized from hot water as leaflets, which blackened without melting on heating to 300°. Its solubility was similar to that of the *ortho* compound and it showed $[\alpha]_D^{14°} - 94°$ ($c = 0{\cdot}95$ in $N/12$ HCl). Found: C, 50·1; H, 5·3; N, 4·9%. $C_{12}H_{15}O_7N$ requires C, 50·5; H, 5·3; N, 4·9%.

p-*Aminophenylglucuronide.* *p*-Aminophenol (1 g.) was fed by stomach tube with water to each of six rabbits. The urine was collected in two portions as before. The first (340 ml.), collected after 3½ hr., was dark but clear. It gave a purple diazo reaction and positive indophenol and naphthoresorcinol reactions. It reduced ammoniacal $AgNO_3$ in the cold, and an ether extract gave the indophenol reaction, indicating the presence of free *p*-aminophenol in the urine (*p*-aminophenylglucuronide is insoluble in ether).

The urine was worked up as in the other two cases and the resulting p-*aminophenylglucuronide* (1·4 g.) was recrystallized from hot water, from which it formed needles, M.P. 213° (decomp.). The second batch of urine was collected up to 24 hr. after feeding the phenol and although some glucuronide was present, it could not be obtained crystalline. The total crystalline material isolated was 1·4 g. or 8·4% of the aminophenol fed. It was insoluble in cold water, ethanol or ether and the analytical figures showed it to contain 1 mol. of water of crystallization, which was not lost at 104° after several hours. It showed $[\alpha]_D^{22°} - 82·7°$ ($c=1$ in $N/10$ HCl). Found: C, 47·6; H, 5·55; N, 4·45%. $C_{12}H_{15}O_7N$, H_2O requires C, 47·5; H, 5·6; N, 4·6%.

Isolation of sulphonamidoaminophenylglucuronides

4-Sulphonamido-2-aminophenylglucuronide. Rabbits were given by stomach tube with water, 0·4–0·6 g./kg. of *o*-aminophenol-*p*-sulphonamide. The urine passed within the first hour gave a red colour in the diazo reaction and a positive naphthoresorcinol reaction, thus indicating excretion of the conjugated glucuronide of the phenol. The urine was collected for 24 hr. In one experiment, for example, a total of 4·4 g. of the sulphonamide was fed and 670 ml. urine were collected in 24 hr. The basic lead precipitate was prepared as usual, the lead removed by H_2S, and the filtrate evaporated to dryness *in vacuo*. The residual syrup taken up in ethanol and again concentrated at 45° crystallized only after the addition of crystals prepared from a small portion dissolved in a little ethanol to which an excess of light petroleum had been added. The yield of glucuronide was 0·42 g. from 4·4 g. of the sulphonamide fed (other preparations yielded 0·2 and 0·21 g. from 3 and 4·2 g. of the sulphonamide, respectively).

2-Sulphonamido-4-aminophenylglucuronide. Three rabbits* were each given 1·3 g. (0·75 g./kg.) of *p*-aminophenol-*o*-sulphonamide [Williams, 1942]. The urine (590 ml.), which was blackish in colour, was collected for 24 hr. and worked up as usual. The non-crystalline concentrate from the basic lead precipitate was dissolved in absolute ethanol, charcoaled, filtered and then concentrated *in vacuo* to a syrup. The solution in aqueous ethanol would not crystallize without the addition of a trace of crystalline 4-sulphonamido-2-aminophenylglucuronide, and 0·62 g. of crystalline 2-*sulphonamido-4-aminophenylglucuronide* was thus obtained. It formed small thick rectangular plates (not notched like its isomeride) from hot water. It did not melt up to 300°, but turned red at 140° and black at 190°. It was almost insoluble in cold water and insoluble in ethanol and ether. It showed $[\alpha]_D^{21°} - 76·9°$ ($c=1$ in water as monosodium salt). Found: C, 35·4; H, 5·1; N, 6·35; S, 7·65%. $C_{12}H_{16}O_9N_2S$, $2\frac{1}{2}H_2O$ requires C, 35·2; H, 5·1; N, 6·8; S, 7·8%.

The action of o-, m- *and* p-*aminophenylglucuronides on haemoglobin solutions*

Human red cells from the blood bank were used. The blood had been treated with Na citrate and allowed to stand for 7 days at a low temperature. Then the plasma had been poured off and the residual red cells were used in these tests. In the first case, 10 ml. of the red cells as obtained from the blood bank were diluted to 200 ml. with water. In the second case, the red cells were washed three times in the centrifuge with 0·9% NaCl, haemolysed by shaking with asbestos and filtered through a thick layer of cotton-wool. 5 ml. of the filtrate were diluted to 200 ml. The results of the experiments were essentially the same for both solutions of haemoglobin. To each of a series of small test tubes containing 3 ml. of the haemoglobin solution was added the required amount of an aqueous solution

Table 1. *Action of* o-, m- *and* p-*aminophenylglucuronides on haemoglobin*

Compound	mg. compound / ml. Hb solution	Time (hr.) 0	0·75	2	3	5–6
Control	0	–	–	–	–	–
o-	3	–	–	+	+ +	.
m-		–	–	–	–	–
p-		–	–	–	–	+
o-	6	–	+	+ +	+ + +	.
m-		–	–	–	–	–
p-		–	.	+	+	+
o-	9	–	+	+ + +	+ + + +	.
m-		–	–	–	–	+
p-		–	.	+	.	+ +
o-	12	–	+ +	+ + + +	+ + + +	.
m-		–	–	–	–	+
p-		–	.	+ +	.	+ + +

– Oxyhaemoglobin spectrum unchanged.
\+ Faint methaemoglobin line in red.
\+ + Definite methaemoglobin line in red.
\+ + + Strong methaemoglobin line in red.
\+ + + + Stronger methaemoglobin line in red.
Blank No observation at that time.

4-Sulphonamido-2-aminophenylglucuronide formed small rectangular notched plates from hot water, M.P. 150° (decomp. to an orange froth). It was almost insoluble in cold water, insoluble in ethanol and ether, but soluble in hot water, acid and alkali. It showed $[\alpha]_D^{20°} - 67·7°$ ($c=2$ in water containing 1 equiv. Na_2CO_3, i.e. enough alkali to form a monosodium salt). Found: C, 35·7; H, 5·1; N, 6·6; S, 7·4%. $C_{12}H_{16}O_9N_2S$, $2\frac{1}{2}H_2O$ requires C, 35·2; H, 5·1; N, 6·8; S, 7·8%.

* Two of these rabbits died 2 days later and the third, being obviously ill, was killed. In all these animals the liver was found to be pulpy and friable. One day after administration of the sulphonamide all three animals salivated profusely.

of the Na salt of the aminophenylglucuronide and water to make up to 5 ml. The tests were set up in duplicate and incubated, with the appropriate controls, at 37°. The tubes were periodically examined with a spectroscope for methaemoglobin. Initially all solutions gave a well-marked oxyhaemoglobin absorption spectrum. The results are given in Table 1.

The aqueous solutions of the Na salts of all three aminophenylglucuronides were subjected to continuous extraction with ether for several hours, but in no case could free aminophenols be detected in the extract. Added free aminophenols could easily be extracted under these conditions.

Table 2. *On methaemoglobin formation* in vitro

Compound	Mg. compound 5 ml. sol.	Time (hr.) 1	1·5	2	3	5·5
Control (water)	0	–	–	–	–	–
Control (Na_2CO_3 solution)	0	–	–	–	–	–
Glucurone	8	–	–	–	–	–
Sulphanilamide	8	–	–	–	–	–
Metanilamide	8	–	–	–	–	–
Orthanilamide	8	–	–	–	–	–
Acetylsulphanilamide	10	–	–	–	–	–
l-Menthylglucuronide	17	–	–	–	–	+
Euxanthic acid	18	–	–	+	+	+
o-Aminophenylglucuronide	14	–	++	+++	++++	++++
m-Aminophenylglucuronide	14	–	–	+	+	+
p-Aminophenylglucuronide	15	–	+	+	++	+++
4-Hydroxy-3-aminobenzenesulphonamide	9	–	–	+	+	++
3-Hydroxy-4-aminobenzenesulphonamide	9	–	–	+	+	+
3-Hydroxy-4-aminobenzenesulphonic acid	9	++	++	++++	++++	++++
6-Hydroxy-3-aminobenzenesulphonamide	9	–	+	+	Solution discoloured	
6-Hydroxy-3-aminobenzenesulphonic acid	9	++	++	++++	++++	++++
4-Sulphonamido-2-aminophenylglucuronide	17	–	+	+	+	+
2-Sulphonamido-4-aminophenylglucuronide	17	–	+	+	+	++
o- and *p*-Aminophenols	5	Solutions discoloured				
m-Aminophenol	5	–	–	–	–	–

– Oxyhaemoglobin spectrum unchanged.
\+ Methaemoglobin spectrum just visible.
++ Methaemoglobin spectrum definite.
+++ Methaemoglobin spectrum strong.
++++ Methaemoglobin spectrum very strong.

The sulphonamidoaminophenylglucuronides and some other compounds were also tested (Table 2). Each compound, in amount equimolar to that of aminophenol (5 mg.), was weighed into a small test tube, each tube being in duplicate. The compound was dissolved with slight warming, if necessary, in 1 ml. Na_2CO_3 containing 1 equiv. (i.e. 2·6 mg./ml.). To each tube were then added 4 ml. haemoglobin solution. Hence the compounds used were present in amounts equivalent to 1 mg. aminophenol/ml. The tubes were incubated and the spectra observed periodically as before.

SUMMARY

1. The preparation is described of *o*-, *m*- and *p*-aminophenylglucuronide and 2-sulphonamido-4-amino- and 4-sulphonamido-2-aminophenylglucuronides by feeding the corresponding aminophenols to rabbits.

2. It has been shown that the *o*- and *p*-aminophenylglucuronides are methaemoglobin-formers *in vitro*. The sulphonamidoaminophenylglucuronides also caused methaemoglobin-formation, but less powerfully than the aminophenylglucuronides.

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The Effect of Citrate on the Rotation of the Molybdate Complexes of Malate, Citramalate and Isocitrate

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Extracts of liver and muscle convert citric acid into laevo-rotatory isocitric acid [Martius, 1938]. As the rotation of isocitric acid, like that of other α-hydroxy acids, is much increased by $(NH_4)_2MoO_4$*, the polarimeter can be used to measure low concentrations of isocitric acid in tissue extracts [Martius, 1938; Jacobsohn, 1940].

On applying this method to plant extracts we met with an unexpected complication. Aqueous extracts of rhubarb petioles showed a dextrorotation with molybdate, owing to the presence of *l*(−)malic acid. When the extracts were mixed with citrate the dextrorotation increased very considerably, e.g. from +1·75° to +3·25°. Investigation of this effect showed that the increase was not caused by an enzymic process; it proved to be due to an effect of citrate on the dextrorotation of the *l*(−)malate-molybdate complex. Under the test conditions $[\alpha]_D^{20°}$ was +716° for *l*(−)malic acid in the presence of $(NH_4)_2MoO_4$ and +1340° for *l*(−)malic acid in the presence of $(NH_4)_2MoO_4$ and citrate. Citrate was found also to change the rotation of the molybdate complexes of two substituted malic acids, viz. citramalic and isocitric acids.

The effect of citrate on the rotation of the malate-molybdate complex has already been observed by Auerbach & Krüger [1923] who realized that citrate interferes with the polarimetric determination of malic acid. Obviously citrate also interferes with the Martius polarimetric isocitric acid estimation. Isocitric acid is frequently accompanied in biological material by citric acid and many of the data calculated from polarimetric readings by previous authors therefore require revision.

EXPERIMENTAL

Materials. Samples of *l*(−)malic acid obtained from Messrs Schering-Kahlbaum and from Dr W. Fraenkel, Factory M3, Treforest Estate, near Cardiff, were used, both giving identical results. Na-*l*(−)α-hydroxyglutarate was prepared according to Fischer & Moreschi [1912], citramalic acid according to Michael & Tissot [1892]; it was resolved with brucine according to Marckwald & Axelrod [1899]; the sample of *l*(+)lactic acid used was obtained from Messrs Schuchardt. Pure optically active isocitric acid was not available; a solution of the active acid was prepared by bacterial resolution of Na isocitrate synthesized according to Fittig & Miller [1889] and Wislicenus & Nassauer [1895].

Calculations. The specific rotation was calculated according to the formula $[\alpha]_D = 100/l \times c$. The values of *c* refer to the concentrations of the free organic acid (g. %), and not to the concentration of the molybdate complexes.

* This formula is used, for brevity's sake, to denote commercial ammonium molybdate.

RESULTS

Effect of citrate on the rotation of extracts of rhubarb petioles

Petioles of forced rhubarb were minced and then ground to a pulp in a mechanical mortar; half volume of H_2O was added during the grinding. Ten ml. pulp, 5 ml. *M* Na_3-citrate and 1 ml. 3 *M* acetate buffer of *p*H 4·0 were mixed and allowed to stand for 19 hr. at 18°, octanol serving as an antiseptic. Then 1·6 ml. glacial acetic acid and 14·4 ml. freshly prepared 29 % $(NH_4)_2MoO_4$ were added, the insoluble material filtered off and the filtrate cleared with a small quantity of charcoal.

A control tube contained water instead of the citrate solution. The polarimeter readings (2 dm. tube, Na-lamp) were: +3·25°; control, +1·75° (18°).

The results were independent of the period of incubation but approximately proportional to the quantity of pulp used. The effect of citrate could not therefore be attributed to the action of an enzyme.

Effect of citrate on the rotation of the molybdate complex of malic acid and other hydroxy acids

The rotation of *l*(−)malic acid (in the presence of $(NH_4)_2MoO_4$) is raised by citrate (Table 1) in the same proportion and in the same direction as is the rotation of the rhubarb extract, the increase being 85·5 % in the case of rhubarb extract and 87 % in the case of malic acid. The effect of citrate on the rotation of rhubarb extract can thus be explained as an effect on the rotation of malate (which is known to occur in rhubarb). The rotation of the molybdate complexes of lactic, tartaric and α-hydroxyglutaric acid are also changed by citrate but these effects are small as compared with the effect of the rotation of malic acid.

Citramalic acid (methyl malic acid) behaves similarly to malic acid. The rotations of the molybdate complexes of malic and citramalic acids are almost identical, both in the absence and in the presence of citrate (Table 1).

Table 1. *Rotatory power of hydroxy acids in the presence of $(NH_4)_2MoO_4$ and other substances*

(2·5 ml. hydroxy acid solution; 2·5 ml. solution of the 'second substance'; 0·5 ml. glacial acetic acid; 4·5 ml. 29% $(NH_4)_2MoO_4$; 18°; 1 dm. tube.)

Hydroxy acid (final conc.)	'Second substance' (final conc.)	α_D (°)	$[\alpha]_D$ (°)
l(−)Malic acid (0·0187 *M* = 0·25%)	—	+1·79	+ 716
	Na_3-citrate (0·25 *M*)	+3·35	+1340
l(+)Lactic acid (0·25 *M*)	—	−1·31	− 58
	Na_3-citrate (0·25 *M*)	−1·28	− 56
l(+)Tartaric acid (0·0334 *M*)	—	+3·30	+ 660
	Na_3-citrate (0·25 *M*)	+3·48	+ 696
l(−)α-Hydroxyglutaric acid (0·026 *M*)	—	−0·39	− 101
	Na_3-citrate (0·25 *M*)	−0·30	− 78
l(−)Citramalic acid (0·01 *M*)	—	−1·11	− 748
	Na_3-citrate (0·25 *M*)	−1·98	−1335
l(−)Malic acid (0·0187 *M*)	Na_3-racemate (0·25 *M*)	+1·21	+ 484
	dl-Na-lactate (0·25 *M*)	+1·31	+ 524
	dl-Na-β-hydroxybutyrate (0·25 *M*)	+1·73	+ 692
	Glycerol (0·6 *M*)	+1·44	+ 576
	Na_3-*trans*-aconitate (0·25 *M*)	+1·79	+ 716
	dl-Na_3-citramalate (0·25 *M*)	+1·84	+ 736

Effects of optically inactive hydroxy compounds other than citrate. Racemic acid, *dl*-lactic acid, *dl*-β-hydroxybutyric acid or glycerol all depress the rotation of the malate-molybdate complex, whilst *trans*-aconitic acid and *dl*-citramalic acid have no definite effect (Table 1).

Effect of citrate concentration. A concentration of citrate equivalent to that of malate already increases the rotation of malic acid considerably, but the maximum effect requires, under the test conditions, a citrate concentration of about 0·1 molar, i.e. about 5 times the concentration of malate. Very high concentrations of citrate (1 *M*) abolish the dextrorotation of the malate-molybdate complex, presumably by competitive reaction with the available molybdate (Table 2).

Table 2. *Effect of citrate concentration on the rotation of the malate-molybdate complex*

(2·5 ml. 1% *l*(−)malic acid solution—final malic acid conc.: 0·0187 *M*; 2·5 ml. Na_3-citrate solution diluted to different conc.; 0·5 ml. glacial acetic acid; 4·5 ml. 29% $(NH_4)_2MoO_4$; 15°; 1 dm. tube.)

Citrate (final conc.) (*M*)	α_D (°)	$[\alpha]_D$ (°)
1·0	−0·08	—
0·5	+0·81	+ 324
0·25	+3·37	+1340
0·125	+3·36	+1335
0·0625	+3·16	+1260
0·03125	+2·82	+1130
0·01563	+2·48	+ 990
None	+1·79	+ 716

Effect of molybdate concentration. The rotation of the malate-molybdate complex does not significantly change when the concentration of $(NH_4)_2MoO_4$ varies between 3 and 13·5%. With citrate there is a diminished rotation when the molybdate concentration falls below 9%. Below this concentration the available molybdate is probably insufficient to 'saturate' both citrate and malate (Table 3).

Table 3. *Effect of $(NH_4)_2MoO_4$ concentration on the rotation of l(−)malic acid in the presence of citrate*

(2·5 ml. 1% *l*(−)malic acid; 0·5 ml. glacial acetic acid; 2·5 ml. 1 *M* Na_3-citrate or water; 4·5 ml. $(NH_4)_2MoO_4$ solution of varying strength; 15°; 1 dm. tube.)

$(NH_4)_2MoO_4$ (final conc.) (%)	α_D without citrate (°)	α_D with citrate (°)
13·5	+1·82	+3·50
9·0	+1·85	+3·37
6·0	+1·87	+0·96
3·0	+1·85	−0·08

Effect of the malic acid concentration. The specific rotation of the malate-molybdate-citrate complex is virtually constant when the malic acid concentration rises from 0·015 to 0·25% (Table 4).

Table 4. *Effect of malic acid concentration on the rotation of the malate-molybdate-citrate complex*

(2·5 ml. malic acid solution; 2·5 ml. Na_3-citrate 1 *M*; 0·5 ml. glacial acetic acid; 4·5 ml. 29% $(NH_4)_2MoO_4$; 17°; 1 dm. tube.)

Malic acid (final conc.) (%)	α_D (°)	$[\alpha]_D$ (°)
0·25	+3·34	+1335
0·125	+1·76	+1407
0·0625	+0·90	+1440
0·03125	+0·45	+1440
0·01563	+0·23	+1470

Effect of temperature on the rotation of the malate-molybdate-citrate complex. At 6·5°, 17·5° and 37·5° we find for α_D +3·44°, +3·34° and +3·03° (under the conditions stated in Table 4).

Effect of citrate on the rotation of the citramalate-molybdate complex. The effect of citrate on the rotation of the citramalate-molybdate complex is similar in magnitude to the effect on the malate complex (Table 5).

Table 5. *Effect of citrate on the rotation of the citramalate-molybdate complex*

(3 ml. 0·445% (−)citramalic acid solution—final citramalic acid conc. 0·01 *M*; 2 ml. Na_3-citrate solutions of varying conc.; 0·5 ml. glacial acetic acid; 4·5 ml. 29% $(NH_4)_2MoO_4$; 22°; 1 dm. tube.)

Citrate (final conc.) (*M*)	α_D (°)	$[\alpha]_D$ (°)
0·2	−2·04	−1380
0·1	−2·00	−1350
0·05	−1·90	−1280
0·025	−1·73	−1170
0·0125	−1·55	−1040
0·00625	−1·41	− 452
0·00313	−1·27	− 856
None	−1·11	− 748

Experiments with isocitric acid

Since pure optically active isocitric acid was not available a solution of the active acid was prepared by bacterial resolution of synthetic Na_3-isocitrate. *Pseudomonas pyocyanea, Aerobacter aerogenes* or *Bact. prodigiosum* all resolve isocitrate [Martius, 1938]. The culture medium contained 1 g. KH_2PO_4, 1 g. Na_2HPO_4, 5 g. NH_4Cl, 0·2 g. $MgSO_4$, $7H_2O$, 0·05 g. $CaCl_2$ and 9·6 g. isocitric acid (as Na salt) per litre. 100 ml. medium were sterilized in Roux culture flasks of 1 l. capacity and inoculated with one of the above organisms. The flasks were laid out flat in order to facilitate aeration. When a heavy inoculum was used the maximum rotation was reached after 3 or 4 days' incubation at 37°, α_D of the medium being +1·69° (2 dm. tube; 5 ml. medium diluted with 0·5 ml. glacial acetic acid and 4·5 ml. 29% molybdate, filtered).

Like muscle tissue [Martius, 1938], the bacteria tested metabolize 50% of synthetic isocitrate. This is shown by the observation that a washed suspension of *Aerobacter aerogenes* (*p*H 6·8; 40°) absorbed 438 μl. extra O_2 on addition of 0·1 ml. 0·05 *M* citrate, and 226 μl. on addition of the same amount of synthetic isocitrate (calc. for complete oxidation: 504 μl.). The fact that less than the calculated amount of O_2 was absorbed was presumably due to the synthesis of cell material from the substrate [cf. Clifton, 1937].

For the following experiments 300 ml. medium were incubated for 3 days with *Aerobacter aerogenes*; the culture was then concentrated on the steam-bath to about 50 ml., and filtered after addition of charcoal. The effect of citrate on the rotation of the isocitrate solution in the presence of molybdate is shown in Tables 6 and 7. The increase caused by 0·1 *M* citrate varies between 40% and 120%.

Table 6. *Rotation of isocitrate solutions of varying concentrations in presence of citrate and molybdate*

(10 ml. isocitrate solution; 1·0 ml. glacial acetic acid; 9 ml. 29% $(NH_4)_2MoO_4$; 2 dm. tube; for the measurements recorded in the last two columns the isocitrate stock solution was diluted with Na_3-citrate solutions; the citrate concentration stated refers to the final concentration.)

Relative conc. of isocitrate (Data are no. of times stock solution was diluted)	α_D (°)		
	No citrate added	0·1 *M* citrate	0·2 *M* citrate
Undiluted	+9·64	—	—
2	+5·62	+7·86	+6·44
4	+2·72	+4·52	+3·96
8	+1·23	+2·47	+2·12
16	+0·58	+1·26	+1·12
32	+0·30	+0·66	—

Table 7. *Effect of citrate concentration on the rotation of the isocitrate-molybdate complex*

(Stock solution of isocitrate diluted with 3 vol. H_2O; 4 ml. diluted stock solution; 1 ml. citrate; 0·5 ml. glacial acetic acid; 4·5 ml. 29% molybdate; 20°; 1 dm. tube.)

Citrate (final conc.) (*M*)	α_D (°)
0·1	+1·89
0·05	+1·77
0·025	+1·58
0·0125	+1·41
0·00625	+1·27
0·003125	+1·11
None	+1·04

Equilibrium between citric, isocitric and cis*-aconitic acids*

Martius [1938] calculated from polarimetric readings that the equilibrium mixture of the three tricarboxylic acids in the presence of aconitase contains 9·4% isocitric acid. The calculation was based on the assumption that the specific rotation of isocitric acid in the presence of molybdate is −428°. Since this value applies to pure isocitric acid solutions it is no doubt too low for the conditions of Martius's experiments, where about 0·09 *M* citrate was present.

The data presented in Tables 6 and 7 permit an approximate estimate of the specific rotation of isocitric acid in the presence of citrate. Under the conditions of Martius's measurements the increase brought about by citrate is about 82%; this would bring $[\alpha]_D$ to −780°. Using this figure for the recalculation of Martius's data we obtain a value of 5·15% isocitric acid in the equilibrium mixture.

A number of further measurements of the final rotation of solutions in which the enzymic equilibrium between the three tricarboxylic acids was established, are shown in Table 8. The solutions

contained 0·2 *M* Na citrate, varying quantities of buffer, minced tissue and a drop of octanol. The mixture was incubated at 38°. For the determination of the rotation 10 ml. were acidified with 1 ml. glacial acetic acid, ice-cooled, and mixed with 9 ml. 29 % $(NH_4)_2MoO_4$. The solution was filtered after adding a small quantity of charcoal, and rapidly examined in 2 dm. tubes. Ice-cooling was necessary in order to prevent the development of the blue colour of the reduced phosphomolybdate complex. Under the conditions of the measurements the concentration of citrate was about 0·09 *M*.

Table 8. *Equilibrium at 38° between citrate, isocitrate and* cis*-aconitate in the presence of aconitase*

(Unless otherwise stated the tissue concentration in the enzyme preparation was 3·0 g. of fresh tissue in 50 ml.; the buffer consisted of phosphate except in the one instance indicated, in which $NaHCO_3/CO_2$ was used.)

Buffer: Final conc. *M*	pH	Period of incubation hr.	$\alpha_D^{18°}$ (° for 2 dm.): Without citrate	With citrate	Difference	*cis*-Aconitic acid %*
			(*a*) Enzyme from guinea-pig liver			
0·030	7·4	18†	+0·06	−1·74	−1·80	4·08
0·025	6·8	17	+0·13	−1·70	−1·83	3·52
0·025	7·1	17	+0·12	−1·68	−1·80	—
0·025	6·8	17	+0·11	−1·79	−1·90	4·19
0·025	6·8	17	+0·12	−1·72	−1·84	5·04
0·050	6·8	17	+0·02	−1·73	−1·75	4·26
0·060‡	6·4‡	24	−0·14	−1·81	−1·67	—
0·025	6·8	17	+0·13	−1·77	−1·90	4·73
0·025	6·8	17	+0·16	−1·72	−1·88	4·22
			(*b*) Enzyme from guinea-pig muscle			
0·010	6·8	41	−0·06	−1·82	−1·76	4·33
			(*c*) Enzyme from pigeon breast muscle			
0·025	6·8	22	−0·01	−1·77	−1·76	4·22

* The figures in this column are

$$\frac{\text{molecules of } cis\text{-aconitic acid found}}{\text{molecules of citric acid added}} \times 100.$$

† Tissue conc. = 1·5 g./50 ml.

‡ Buffer = $NaHCO_3/CO_2$.

The rotations observed were a little higher than those reported by Martius [1938] and by Jacobsohn, Soares & Tapadinhas [1940]. The average of six measurements of α_D under the same conditions (*p*H 6·8; 0·025 *M* phosphate) is −1·85°. The deviations from this mean are considerably greater than the experimental error (which is ±0·04°). They may be connected with the rotation of the control. As the crude tissue extracts used in our experiments contained cathepsin and amylase, optically active substances may have been formed during the incubation from protein or carbohydrates. It is possible that the high concentration of citrate affects these enzymes and this would invalidate the control experiments. More accurate estimations of the rotation caused by isocitric acid would require the use of a purified aconitase free from other enzymes and their substrates.

On the basis of the assumption that $[\alpha]_D$ for the experimental conditions is −780°, the rotation of −1·85° indicates that 6·2 % of the citric acid was converted into isocitric acid. The average proportion of aconitic acid (determined according to Johnson [1939]) was 4·3 %. This leaves 89·5 % for citric acid. Determinations of citric acid according to Pucher, Sherman & Vickery [1936] and Kometiani [1931] agreed with this calculation within the limits of the error of the methods, which proved to be about ±10 %.

The values for *cis*-aconitic acid are in accordance with those previously reported from this laboratory by Johnson [1939]. Johnson calculated the isocitric acid concentration of the equilibrium mixture by deducting the sum of the aconitic and citric acid recovered from the amount of citric acid added. In the light of more recent experience with the citric acid determination according to Pucher *et al.* [1936] [see also Dickens, 1941], it is probable that Johnson's recovery of citrate was somewhat incomplete. We find that the yield is frequently low when permanganate is added rapidly to the citrate solution and we therefore now add the reagent gradually, as recommended by Reichard [1934]. This precaution was not observed in Johnson's experiments; his citrate values therefore were probably too low, and the calculated isocitrate values too high.

Change of *p*H from 6·8 to 7·4, or the substitution of phosphate buffer by bicarbonate buffer, does not appreciably affect the equilibrium (Table 8). Confirming Jacobsohn's [1940] observation we find that addition of $MgCl_2$ (0·12 *M*) reduces the concentration of isocitric acid in the equilibrium mixture from 6·2 to 2·6 % (assuming $[\alpha]_D$ to be −780°). Under the same conditions (*p*H 6·8; 0·025 *M* phosphate buffer) the concentration of *cis*-aconitic acid fell from 4·2 to 1·6 %. $MgCl_2$ thus shifts the equilibrium in favour of citrate, presumably because of the formation of a Mg-citrate complex [see Hastings, McLean, Eichelberger, Hall & da Costa, 1934; Nordbö, 1938] which results in a lowering of the concentration of 'free' citrate in the system.

Polarimetric determination of malic and isocitric acids

To overcome the difficulty arising from the interference by citrate with the rotation of the molybdate complexes, we add citrate to the solution in quantities sufficient to bring the citrate concentration to about 0·1 *M*; 4 ml. of the solution to be examined (which should be neutral) are mixed with 1 ml. *M* Na_3-citrate, 0·5 ml. acetic acid and 4·5 ml. 29 % $(NH_4)_2MoO_4$. The data recorded in Tables 4 and 6 show that the rotations of malic and isocitric acids under these conditions are approximately proportional to the hydroxy acid concentration. This procedure has the further advantage of increasing the sensitivity of the polarimetric method.

SUMMARY

1. Citrate increases the rotation of the molybdate complexes of malic acid (as already observed by Auerbach & Krüger [1923]), of citramalic acid and of isocitric acid. The increase can amount to more than 100%. The magnitude of the effect under varying conditions is investigated.

2. The effect of citrate must be taken into account when the above acids are determined polarimetrically by the molybdate method. Isocitric acid concentrations calculated from polarimetric readings by previous investigators who were unaware of the citrate effect require revision.

3. Preliminary experiments show that the equilibrium mixture of isocitrate, *cis*-aconitate and citrate in the presence of liver or muscle aconitase contains 6·2% isocitrate, 4·3% *cis*-aconitate and 89·5% citrate (38°; *p*H 6·8; 0·025*M* phosphate buffer). The effect of *p*H is small between 6·8 and 7·4. $MgCl_2$ shifts the equilibrium in favour of citrate.

4. A modified polarimetric method is suggested for the determination of malic and isocitric acids, applicable to solutions containing citrate.

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Aerobic Oxidation of Aromatic Hydrocarbons in the Presence of Ascorbic Acid

THE REACTION WITH ANTHRACENE AND 3:4-BENZPYRENE

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It has long been known that oxidation of the aromatic hydrocarbons plays a fundamental part in their elimination from the animal body, but isolation of the products excreted has contributed little to knowledge of the initial introduction of oxygen into the molecule. This problem has assumed even greater importance recently in connexion with the fate of carcinogenic hydrocarbons in the animal body. Many such hydrocarbons are now known and, in most cases, introduction of oxygen into the molecule leads to considerable or complete loss of carcinogenic activity. Thus in these carcinogens oxidation is equivalent to 'detoxication' from the point of view of cancer induction.

It is well known that the feeding of naphthalene to rabbits leads to the production of cataract. The crystalline lens normally contains much ascorbic acid. Simultaneously with the development of opacity in the lens during cataract formation the ascorbic acid content falls to a very low value. It has also been reported that the administration of ascorbic acid to animals on a naphthalene diet inhibits cataract production. It must be admitted that the whole question of the relation of ascorbic acid to cataract production by naphthalene is still undecided, and there is no general agreement on the facts. However, these experiments suggested to Jorissen [1937] the desirability of testing the

action of ascorbic acid on naphthalene *in vitro*. In the aqueous acetone solution of naphthalene containing ascorbic acid, he detected the formation of an oxygen-containing derivative of naphthalene which he assumed to be a naphthol.

This observation has been studied in more detail in the present work and extended to other hydrocarbons. Evidence of oxidation of the hydrocarbon has been obtained with naphthalene, phenanthrene, anthracene, and with the carcinogenic hydrocarbons 3:4-benzpyrene, cholanthrene, and methylcholanthrene. Experiments on the identification of the products from anthracene and 3:4-benzpyrene are described.

EXPERIMENTAL

The technique was very simple. The hydrocarbon was dissolved in a suitable volume of 80 % acetone (B.D.H. Analar) and 2–4 times its weight of ascorbic acid added. The flasks, which were large enough to contain ample air, were stoppered and kept in the dark at room temperature (usually about 20°). Light was excluded to avoid photo-oxidation.

Anthracene

Examined after 2 weeks, solutions containing anthracene and ascorbic acid showed no obvious difference in colour when compared with similar control solutions containing no ascorbic acid. Water was added to the solution and the acetone removed under reduced pressure at a low temperature. The solid material which separated (mainly unchanged anthracene) was collected and well washed with water. This residue was heated with *N* NaOH and Zn dust. The filtrate from Zn and unchanged hydrocarbon was deep red. The extraction with alkali and Zn was repeated until the filtrates and washings were colourless. On aeration of the alkaline filtrate a brownish solid separated and the solution lost its deep red colour. The solution was made slightly acid and the precipitate collected and dried. Recrystallization from glacial acetic acid gave fine yellow needles, M.P. 279–280° (uncorr.), which did not depress the M.P. (279–280°) of authentic anthraquinone.

In solutions containing anthracene and ascorbic acid left for longer periods (2–3 months) crystals of anthraquinone were seen to form as long very pale yellow needles, which on removal and recrystallization had M.P. 279–280°. No anthraquinone could be detected in the control experiments.

3:4-Benzpyrene

This hydrocarbon invariably showed a most striking and characteristic colour change. Benzpyrene in aqueous acetone kept in the dark maintains its pale yellow-green colour even after 5 years. In the presence of ascorbic acid the solution rapidly becomes deep yellow, then orange and finally ruby red. The change is detectable in a few hours, and after standing overnight it is quite obvious.

Isolation of benzpyrene quinones. After removal of the acetone under reduced pressure the precipitated solid was collected by filtration. This solid consisted mainly of unchanged hydrocarbon impregnated with some coloured material which gave the mass a magenta colour. Treatment of this product with 2*N* NaOH in the cold gave a dark alkaline solution with a strong green fluorescence. Acidification of this solution led to its decolorization with precipitation of a small quantity of red flocculent material. The nature of this material is discussed later.

The oxidation product was repeatedly leached with hot 2*N* NaOH until the extracts had lost all the green fluorescence and were practically colourless. Boiling the extracted residue with more 2*N* NaOH and Zn dust led to a red-brown vat. Unchanged hydrocarbon was removed by filtration and the alkaline solution aerated. A red precipitate separated and the solution became very pale green. After acidification the precipitate was collected and dried, when it was obtained as a brick-red powder. The yield was low—*ca.* 10 mg. from 100 mg. of benzpyrene. The melting-point after recrystallization from xylene was 230°, not depressed by admixture with a specimen of 3:4-benzpyrene quinone M.P. 230° [Cook & Hewett, 1933].

Vollmann, Becker, Corell & Streeck [1937] showed that the quinone described by Cook & Hewett is a mixture of 5:8- and 5:10-quinone. Separation was achieved by conversion of the mixture of quinones to the leucodiacetates and fractional crystallization. The 5:10-quinone crystallizes in golden orange needles M.P. 295° and dissolves in concentrated H_2SO_4 to give a carmine red solution. The 5:8-quinone forms red needles M.P. 245° and its solution in conc. H_2SO_4 is olive brown.

The product from ascorbic acid oxidation of benzpyrene gave a solution in conc. H_2SO_4 intermediate in colour between solutions of the pure quinones and indistinguishable from that of a solution of the mixed quinones of Cook & Hewett.

Other products of the oxidation. As mentioned above, some material is extractable from the oxidation product by alkali alone. Since this material can be apparently completely removed before the quinones are extracted by vat formation, it is not quinonoid but presumably phenolic in nature. On precipitation with acid from its alkaline solution it is red and flocculent, but on collection and drying it darkens. All efforts at purification of this material have so far been unsuccessful and have yielded nothing but further small quantities of quinone.

Colorimetric measurements on the oxidation product. These were made by means of a Hilger Photoelectric Absorptiometer using Ilford 'Spectrum' filters. The colour developed in mixtures of benzpyrene and ascorbic acid solution was measured and analysed by using the filters at intervals during 58 days. A typical result is shown in Fig. 1, in

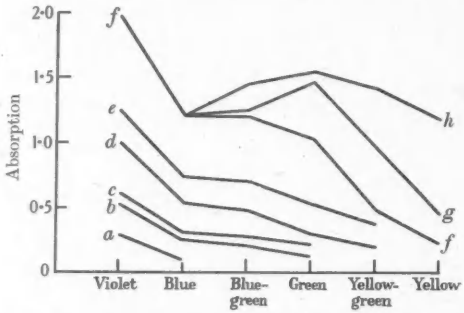


Fig. 1. Colour development in a solution of 3:4-benzpyrene during aerobic oxidation in the presence of ascorbic acid. $a=27$, $b=50$, $c=74$, $d=98$, $e=144$, $f=214$, $g=339$ hr. $h=58$ days.

which are plotted the values obtained by measurement of a solution initially containing 50 mg. benzpyrene and 200 mg. ascorbic acid. Similar measurements were carried out on pure specimens of the 5:8- and 5:10-quinone and on the mixture prepared by chromic acid oxidation of the hydrocarbon (Table 1). Attention may be directed to has fallen by 20 %. This change is not shown by the 5:10-quinone where the absorptions of saturated solutions in pure acetone or in 80 % aqueous acetone are practically indistinguishable.

Comparison of the values shown in the table with those for the ascorbic acid-hydrocarbon experiment given in Fig. 1 indicate that the colour developed in the latter is mainly due to formation of the quinones but that there is also some other chromogen present. In the later curves in the figure the absorption towards the red end of the spectrum is greater than can be accounted for by assuming a saturated solution of the mixed quinones, even if the more chromogenic 5:8-compound predominated.

The influence of various factors on the oxidation. By making use of the rapid development of colour with benzpyrene it was possible to examine the influence of various factors on the speed of the reaction. At temperatures of 30–40° the reaction proceeded more rapidly than at 20–25°, but measurements of the colour produced showed that the reaction at the lower temperatures, although initially slower, finally developed more colour. This is to be expected since the rate of destruction of the ascorbic acid is much greater at the higher temperature range. The rate of reaction was not appreciably increased by vigorous shaking in air or O_2, but the reaction is completely inhibited if O_2 is excluded. Mixtures of ascorbic acid and benzpyrene in aqueous acetone in sealed tubes from which all air has been removed or replaced by N_2 remain colourless for months if they are not exposed to

Table 1. *Absorption measurements on quinones*

Filter ...	V.	B.	B.-G.	G.	Y.-G.	Y.	O.	R.
5:10-Quinone (100 % acetone)	∞	1·07	0·47	0·12	—	—	—	—
5:10-Quinone (80 % acetone)	∞	1·03	0·47	0·11	—	—	—	—
5:10-Quinone (80 % acetone) + ascorbic acid	∞	1·20	1·10	0·65	0·20	—	—	—
5:8-Quinone (100 % acetone)	∞	1·22	1·22	1·06	0·17	—	—	—
5:8-Quinone (80 % acetone)	∞	1·18	1·20	1·50	0·48	—	—	—
5:8-Quinone (80 % acetone) + ascorbic acid	∞	1·23	1·28	1·55	1·15	0·53	0·185	0·06
Mixed quinone in 80 % acetone	∞	1·28	1·28	1·05	0·30	—	—	—

The values = absorption coefficient. Cell depth, 1·0 cm. Concentration—saturated at 15° C.

two points. One is the increase in intensity of the colour of the quinone solutions, particularly towards the red end of the spectrum, when ascorbic acid is present in their solutions. This is especially marked in the case of the 5:8-quinone where the absorption, for example, in the yellow-green is increased from 0·48 to 1·15 on adding ascorbic acid and allowing to stand for several days. The second point is that on diluting the 100 % acetone solution of the 5:8-quinone with water in order to obtain an 80 % solution there is an increase in the absorption intensity although the concentration of the quinone

bright light. On opening the tubes the usual sequence of colour changes takes place.

Since it appeared probable that the autoxidation of the ascorbic acid played an essential part in the mechanism of the oxidation, the effect of copper, cyanide, metaphosphoric acid, and H_2O_2 on the reaction was tried.

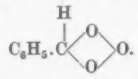
Small traces of cupric ions (1–2 μg./ml.) either had no effect or, occasionally, produced a slight acceleration of the reaction. $M/100$ KCN or 5 % HPO_3, however, completely inhibited the reaction. In view of the possibility that the oxidation of the

hydrocarbon might be due to H_2O_2 formed during autoxidation of the ascorbic acid, experiments were carried out in which H_2O_2 was added to the hydrocarbon-ascorbic acid mixture, and also some in which H_2O_2 alone without ascorbic acid was employed. The addition of the peroxide was without effect on the reaction when both it and ascorbic acid were present in the solution. Alone, the H_2O_2 did not oxidize the hydrocarbon.

These results indicated that the enediol grouping of ascorbic acid was the essential structural requirement for bringing about the oxidation of the hydrocarbon. Support for this view was obtained in experiments in which ascorbic acid was replaced by dihydroxymaleic acid. Exactly similar oxidations of hydrocarbons were obtained by the use of this acid in aqueous acetone solution.

DISCUSSION

The oxidation of aromatic hydrocarbons in the presence of substances undergoing autoxidation is well known. In a study of the inhibitory action of anthracene on the autoxidation of benzaldehyde Bäckström & Beatty [1931] have shown that the inhibitory action is connected with an induced oxidation of the inhibitor. The primary oxidation product is anthranol which is autoxidizable and reacts with oxygen to give a peroxide which is slowly decomposed with the formation of the final reaction product, anthraquinone. The course of the reaction indicated that the initial oxidation of the hydrocarbon is the result of a reaction with a peroxide of benzaldehyde of the structure



Wasley & Rusch [1942] showed that the autoxidation of benzaldehyde and of heptaldehyde was inhibited by anthracene, 3:4-benzpyrene, 1:2:5:6-dibenzanthracene, and 20-methylcholanthrene. In the case of benzpyrene, measurements of absorption spectra indicated that the inhibition involved the oxidation of the hydrocarbon to one or more quinones.

It seems probable that a similar mechanism underlies the reaction described in the present paper. The autoxidation of ascorbic acid may be assumed to involve the formation of a peroxide. If this peroxide oxidizes anthracene to anthranol and 3:4-benzpyrene to 5-hydroxy-3:4-benzpyrene as the initial products, the formation of anthraquinone and a mixture of benzpyrene quinones together with some monohydroxybenzpyrene is readily understood.

Whether such a mechanism plays any part in the *in vivo* oxidation and elimination of aromatic hydrocarbons remains undecided. Experiments on this point, and investigations of the possibility that aromatic hydrocarbons interfere with the normal utilization of ascorbic acid in the animal body, are in progress.

SUMMARY

Oxygen oxidizes aromatic hydrocarbons in aqueous acetone solutions containing ascorbic acid. Anthracene is oxidized to 9:10-anthraquinone and 3:4-benzpyrene yields a mixture of 3:4-benzpyrene-5:8-quinone and 3:4-benzpyrene-5:10-quinone. The oxidizing agent appears to be one of the products formed during autoxidation of ascorbic acid and is possibly dehydroascorbic acid or a peroxide thereof. The mechanism seems to be similar to that which occurs during the inhibition of the autoxidation of aldehydes by aromatic hydrocarbons.

I should like to express my thanks to the Sir Halley Stewart Trust for a Fellowship held during the course of this work. The British Empire Cancer Campaign, in addition to generous support of this Institute, put at my disposal a supply of 3:4-benzpyrene used in this investigation.

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The Action on Avian Malaria of the Alkaloids of Cinchonas from the Cameroons and the Belgian Congo

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During the course of the war it has become increasingly important to seek efficient and cheap remedies against malaria among the alkaloids of the cinchonas.

Numerous experiments on cinchona febrifuges have been carried out in several countries, e.g. Ejercito & Santos [1937], Field [1934], Fourneau, Trefouel, Trefouel, Stefanopoulo, Benoit, Lestrange & Melville [1930], Hicks & Chand Diwan [1935], Kingsbury [1937], League of Nations [1934; 1937], Manon, Perez & Russell [1935], Miyahara [1938], Parvulescu & Boeriu [1934], Sergent, Sergent & Vogt [1925], Sinton [1929], Slatineanu, Ciuca, Balteanu, Alexa, Francke & Rugina [1934], Wijerana [1939], Yao & Yung [1934]. From all these experiments it appears that in the treatment of ordinary cases of malaria cinchona febrifuges are equal to, if not of slightly superior value to, quinine. Nevertheless these cinchona febrifuges vary largely in composition, in origin and in the mode of preparation, and it seems desirable at the present time to obtain more detailed and accurate information about the nature of the total alkaloids from different cinchonas, and to examine their therapeutic action, before considering their intensive cultivation and the establishment of small plants for the purpose of extracting these alkaloids on the spot by simple processes.

The initial experiments to be described are confined to the action on avian malaria of alkaloids from the barks of *C. succirubra* and *C. Ledgeriana* from Dschang (French Cameroons) and Kivu (Belgian Congo). Some experiments on avian malaria have already been carried out by several authors: Bishop [1942]; Buttle, Henry & Trevan [1934]; Buttle, Henry, Solomon, Trevan & Gibbs [1938]; Giemsa [1933]; Goodson, Henry & Macfie [1930]; Sergent, Sergent & Vogt [1923; 1924; 1925]. The alkaloids used in the present study were either extracted by the classical laboratory method or by industrial processes. The samples were in all cases prepared in our laboratories and were practically pure (95–97 %), containing the same percentage of quinine as the laboratory product. Some small differences may exist in the relative proportions of the amorphous alkaloids present.

EXPERIMENTAL

The malaria parasites used in this work were a strain of *Plasmodium relictum* obtained from Dr Keilin of Cambridge to whom we wish to offer our thanks. The birds used were all canaries; owing to difficulties in obtaining supplies these were not all of a constant strain, but gave a uniform response to the infection.

The malaria was transmitted by direct blood inoculation from bird to bird. Blood was obtained from the vein running down the inside of the leg of an acutely infected bird and was collected in an equal volume of citrate-saline (NaCl 0·8 %, Na citrate 2 %). Each bird to be inoculated received 0·25 ml. diluted blood, injected into the pectoral muscle. Usually the examination of the blood of infected birds was begun on the fifth day, the blood films being treated with Giemsa's stain. At least 30 fields were examined on each slide.

The alkaloids were administered orally to the birds in the form of small pellets, which were prepared by mixing 1 part of alkaloid with 9 parts of corn flour, together with a little H_2O. Each pellet thus contained the amount of alkaloid (in form of insoluble base) required for a test on a single bird. The pellets were readily swallowed, and experience has shown that this method was more convenient than administration by catheter [Fourneau *et al.*, 1930].

The routine method of testing compounds was as follows: 6 hr. after a bird had been inoculated, a dose of alkaloid was given and five further doses were given at 24-hr. intervals. There were two untreated control birds in each experiment.

The birds chosen for each test were of approximately equal weight (to within 2 g.) and uniform doses were administered. It was found that under these conditions, the appearance of parasites in the blood is not dependent on the weight of the bird, either in untreated controls or in treated birds.

Most of the experiments were carried out with a daily dose of 3·5 mg. of total alkaloid, an amount which gave a good therapeutic effect without toxic symptoms.

RESULTS

The therapeutic effect of the alkaloids is measured in terms of the retardation of infection in the different groups. The results of experiments are summarized in Tables 1 and 2. In only one bird of the whole series (30 mg. of alkaloid from *C. succirubra*) was the onset of malaria indefinitely retarded.

(ii) *Total alkaloids minus quinine.* Three of the industrial preparations have been tested after the removal of their contained quinine. In a dosage of 3·5 mg./day they produced a retardation of avian malaria for 4–5 days, compared with the figure 7·35–10·5 days observed with the similar dose of alkaloids including quinine. The explanation of this difference may lie in a synergistic action of small amounts of quinine and other alkaloids from cinchonas, and experiments are in progress to elucidate this very important question.

The alkaloids from *C. Ledgeriana* in doses of 2·5 and 3·5 mg. were more effective than the same amount of quinine.

In all the experiments no differences were observed between the industrial and laboratory preparations from the different sources.

Table 1. *Influence of alkaloids* per os *on the time of onset of avian malaria*

Alkaloid	Source of alkaloid: Country	Source of alkaloid: Bark	Method of extraction	Influence of alkaloid on onset of malaria in canaries: Daily dose given (mg.)	Influence of alkaloid on onset of malaria in canaries: No. of birds	Influence of alkaloid on onset of malaria in canaries: Period of delay in onset of malaria (days)
Quinine (base)	—	—	—	2·5	4	5·25
				3·5	6	9·40
				5·0	4	11·00
Total	Cameroons	*C. succirubra*	Laboratory	2·5	4	4·25
				3·5	9	7·35
				5·0	4	>15·20
Total	Cameroons	*C. succirubra*	Industrial	2·5	3	3·70
				3·5	2	7·50
Total	Cameroons	*C. Ledgeriana*	Laboratory	3·5	5	10·20
Total	Cameroons	*C. Ledgeriana*	Industrial	2·5	3	8·00
Total (Sample R)	Belgian Congo	*C. succirubra*	Laboratory	2·5	4	8·50
Total (Sample M)	Belgian Congo	*C. succirubra*	Industrial	2·5	4	9·00
Total minus quinine (Sample E)	Belgian Congo	*C. Ledgeriana*	Industrial	2·5	2	4·50
Total minus quinine	Cameroons	*C. succirubra*	Industrial	2·5	1	4·50
Total minus quinine	Cameroons	*C. Ledgeriana*	Industrial	2·5	2	4·00

Table 2. *Composition of the total alkaloids tested*

Source and method of extraction	Total alkaloid in barks (%)	Composition (%) of total alkaloid: Quinine base	Cinchonidine	Cinchonine	Amorphous alkaloids	Impurities
C. succirubra (Dschang). Industrial	6·40	33	27	14	26	3
C. Ledgeriana (Dschang). Industrial	7·50	62	—	—	—	1
C. succirubra; sample R (Kivu). Laboratory	8·35	22	—	—	—	—
C. succirubra; sample M (Kivu). Industrial	7·00	13	—	—	—	5
C. Ledgeriana; sample E (Kivu). Industrial	7·25	59	—	—	—	—

DISCUSSION

(i) *Total alkaloids (including quinine).* The results indicate that the total alkaloids from all the sources examined have an effect similar to that of quinine in retarding the onset of avian malaria. The effect appears to vary largely according to the origin of the barks—the Belgian *C. succirubra* (M and R) gave a mixture of alkaloids which, although less rich in quinine, seemed to be more effective than the mixture extracted from the French bark.

The alkaloids of *C. succirubra*, when given in doses of 3·5 mg./day, were somewhat less effective than the same amount of quinine base, but on increasing the dose to 5 mg./day, these alkaloids (from the Cameroons) exerted a more powerful therapeutic action than quinine.

SUMMARY

1. The total alkaloids from *Cinchona succirubra* and *C. Ledgeriana* from both the Cameroons and the Belgian Congo, have been tested for therapeutic action on avian malaria.

2. In all cases the results indicate that the total alkaloids have an effect similar to, or even better than, that of quinine.

3. Industrial and laboratory preparations are of equal value.

4. The total alkaloids from which the quinine has been extracted have a poor therapeutic effect, and the difference between these and the results with the total alkaloids may be due to the synergistic action of small amounts of quinine and other alkaloids.

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The Reducing Properties of Fibrinogen

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The protein, fibrinogen, is characterized by (1) its low solubility in moderately strong salt solutions, (2) its conversion to fibrin by thrombin and (3) its marked instability and ease of denaturation. The last property has made difficult its purification and study but it is now possible [Florkin, 1930] to prepare fibrinogen free from other proteins and without any evidence of denaturation. Study of the clotting of this purified fibrinogen by thrombin [Jaques, 1938] could not be continued until a method was found to stop the reaction quickly in order to obtain samples for analysis. The methods commonly used in enzyme chemistry for this purpose (heat, etc.) are inapplicable because they affect the solubilities of fibrinogen and fibrin. Dilute iodine, potassium permanganate and potassium dichromate, tested for this purpose, were found to give a heavy precipitate with fibrinogen. As this phenomenon was not found with the serum proteins and does not appear to have been reported before, it was investigated. Secondary reactions took place with $K_2Cr_2O_7$ and $KMnO_4$, as shown by re-solution of the precipitate. The study was therefore limited to the action of iodine and H_2O_2.

EXPERIMENTAL

Materials

Fibrinogen. Preparation by repeated precipitation with concentrated salt solutions is not very satisfactory in practice. The principle of Florkin [1930], who found it necessary for preparation of homogeneous solutions of the protein that all operations should be made at 2° and at *p*H 6·0–7·0, has been followed. Fibrinogen is rapidly denatured by acid even at low temperatures [Fay & Hendrix, 1931].

Florkin used NaCl as the precipitant, but the resulting 'salt of fibrinogen' tends to form gels and it is difficult to

control the rates of stirring and of addition of the salt, so as to obtain precipitates which dissolve readily. Also, the NaCl used must be carefully decalcified or the fibrinogen will clot after the first precipitation. Fibrinogen precipitated with ammonium and potassium sulphates or phosphates flocculates and has not the same tendency to form gels [cf. Ferguson & Erickson, 1939]. Instead of $(NH_4)_2SO_4$ [Ferguson & Erickson, 1939], phosphate buffer has the advantages that the *p*H is exactly controlled at all stages and the protein content of the solutions can be easily determined by nitrogen analyses. Butler & Montgomery [1932; 1935] have drawn attention to the particularly valuable properties of the equimolecular mixture of K_2HPO_4 and KH_2PO_4, *p*H 6·6, used by Florkin and other associates of Cohn. The method of Florkin has been found by the author to be facilitated if this phosphate buffer is substituted for NaCl. The 3*M* phosphate buffer of Butler & Montgomery is diluted to 2*M* before use, and the fibrinogen is precipitated by adding 1 vol. of this solution to 1 vol. plasma. The concentration of phosphate (*M*) appears to correspond to $\frac{1}{2}$ sat. NaCl solution in its precipitating power. The fibrinogen is washed with *M* phosphate buffer and redissolved in a vol. of *M*/4 phosphate buffer slightly less than that of the original plasma. Three such successive precipitations are made. All operations are conducted in the cold room at 2°. All solutions and glassware are chilled before use and it is advisable to rinse the glassware with a weak solution of Na citrate. The salt solution is added slowly to the continuously stirred solution exactly as directed by Florkin. The use of a centrifuge or a Sharples Super Centrifuge installed in the cold room will be found much preferable to filtration. With these modifications, the whole preparation can be readily carried out in 24 hr. The fibrinogen used in this study was prepared from citrated horse plasma, kindly supplied by Dr E. G. Kerslake of the Connaught Laboratories, University of Toronto.

A simple test to demonstrate the complete absence of other plasma proteins in the fibrinogen solution is the lack of any antithrombin action of heparin when tested with the fibrinogen [Jaques & Mustard, 1940]. Mere traces of plasma are sufficient to restore the antithrombin action of heparin.

Methods

The fibrinogen was dissolved in *M*/4 K phosphate buffer (*p*H 6·6). The reaction vessels were 25 ml. glass-stoppered Erlenmeyer flasks rocked in a thermostat at 25°. 1 ml. fibrinogen solution (0·5 mg. N) + 4 ml. of H_2O were taken and 1 ml. *N*/75 I_2 in KI or H_2O_2 added. The consumption of I_2 by the blank was very small but a control with phosphate buffer instead of the fibrinogen solution was always made. The value reported for the I_2 or H_2O_2 consumption represents the difference between the titre of the control and the titre with fibrinogen added.

The reaction was stopped by adding 1 ml. of 60% KI. The residual I_2 was then titrated with standard *N*/200 $Na_2S_2O_3$ using a 2 ml. microburette and starch as an internal indicator. Residual H_2O_2 was estimated by titrating the equivalent I_2 liberated from the KI. For this, 1 ml. of 4*N* H_2SO_4 was added at the same time as the KI, and after 5 min. the contents were titrated. Clotting was carried out as by Jaques [1938]. The thrombin had been purified by the methods of Seegers and was kindly supplied by Parke, Davis & Co., Detroit.

RESULTS AND DISCUSSION

Kinetics of the oxidation of fibrinogen by H_2O_2 or I_2

The value of *K*, the velocity constant of a bimolecular reaction, was calculated from the data of Table 1 by the equation:

$$K = \frac{1}{t\,(C_A - C_B)} \log_e \frac{(C_A - C_x).C_B}{(C_B - C_x).C_A}.$$

The values of *K* for the reaction with H_2O_2 show very good agreement. This indicates that the reaction is bimolecular and shows that probably a specific reacting group is involved. The values with I_2 do not show such good agreement. This was

Table 1. *Reaction of fibrinogen with H_2O_2 and I_2*

H_2O_2: 1 ml. *N*/100 H_2O_2 + 2 ml. fibrinogen (0·890 mg. N)			I_2: 1 ml. *N*/75 I_2 + 1 ml. fibrinogen (0·500 mg. N)		
Reaction time hr.	H_2O_2 consumed ml.	*K*	Reaction time min.	I_2 consumed ml.	*K*
0·5	0·164	0·00430	8	0·511	0·0179
1·0	0·263	0·00398	25	1·044	0·0179
2·0	0·443	0·00428	48	1·250	0·0141
3·0	0·535	0·00414	75	1·381	0·0153
5·0	0·629	0·00378	79	1·435	0·0132
6·6	0·762	—	120	1·562	0·0126
8·0	0·763	—	300	1·713	—
22·0	0·763	—	720	1·711	—
C_A = 1·953 C_B = 0·763			C_A = 2·750 C_B = 1·712		
K (average) = 0·00410			*K* (average) = 0·0152		

(All concentrations are expressed as equivalent ml. of *N*/200 $Na_2S_2O_3$.)

partly due to technical difficulties but, as indicated later, the reaction with I_2 probably involves several groups. The results with I_2 indicate, however, that here also the reaction involves specific groups of the protein. The velocity of the reaction is fairly high, e.g. with I_2 equilibrium was reached in 5 hr. This was in the presence of 0·06% KI from the standard I_2 solution, and KI strongly inhibits the reaction. At equilibrium, 2·17 mg. I_2 and 0·146 mg. H_2O_2 were reduced per mg. fibrinogen. The same equilibrium values were obtained when the relative concentrations of fibrinogen and oxidizing agent were varied. Iodine evidently takes the oxidation further than H_2O_2: the I_2 consumed is just 4 times the H_2O_2—at equilibrium 3·42 ml. *N*/200 I_2 compared with 0·856 ml. *N*/200 H_2O_2/mg. N.

The effect of various factors on the reaction is shown in Fig. 1: (*a*) varying amounts of KI, followed by 1 ml. *N*/75 I_2, were added to a series of tubes and the tubes allowed to stand 7 hr. KI strongly suppresses the reaction, a final concentration of 6% being sufficient to inhibit it completely.

(*b*) Fig. 1*b* shows the effect of *p*H. The amount of I_2 reduced in 1 hr., and of H_2O_2 in 5 hr., was determined. The *p*H level was established by adding before the oxidizing agent either *N*/2 H_3PO_4 or *N*/2 KOH to the fibrinogen solution. From zero reduction at *p*H 2·3, the amount of I_2 reduced rose rapidly with increasing *p*H. Beyond *p*H 7·5 the 'blank' consumption of I_2 became so great that it interfered with the titrations, but there was no evidence of a decreased consumption by fibrinogen. The effect of *p*H on the reduction of H_2O_2 is similar to but less than the effect on I_2. There appears to be a definite break in the *p*H-curve at the isoelectric point of fibrinogen, the H_2O_2 consumption increasing only relatively slowly on its alkaline side. Isoelectric precipitation of fibrinogen occurred from *p*H 4·4 to 5·4. With I_2, precipitation occurred from *p*H 3·1 to 7·5. With H_2O_2, precipitation was observed only in the region of the isoelectric point of fibrinogen. (*c*) Fig. 1*c* shows the I_2 consumption found with varying amounts of fibrinogen, the phosphate concentration being always *M*/12. The I_2 consumption was determined after 1 hr. The resulting curve approximates to a straight line. The approximately linear relationship and the close agreement observed with duplicates (Fig. 1*c*) suggest that the reaction might be used for the determination of fibrinogen. When the oxidation proceeded for only ½ hr., the graph tended to become parabolic.

The results obtained with I_2 (inhibition by KI, rapid increase in the amount of I_2 reduced, with rise in *p*H, etc.) closely resemble those obtained in the iodimetric determination of cysteine and related compounds [Lucas & King, 1932] and suggest that sulphydryl group is responsible. Fibrinogen does not contain a free SH group, however, for it gives a negative nitroprusside reaction. Very little iodine substitution has occurred in the protein, as shown by analysis. It appears likely, therefore, that the reduction of I_2 and H_2O_2 by fibrinogen is due to a group other than sulphydryl.

The reaction of the serum proteins with I_2

A qualitative difference between the reactions with serum proteins and with fibrinogen is evident from the fact that the former do not give any precipitate with I_2. The I_2 consumption of a sample of horse plasma was therefore determined as usual and was corrected for the reducing power of the fibrinogen present. 0·10 ml. plasma (1·10 mg. N) gave an I_2 uptake for 1 hr. of 1·964 ml. Total fibrinogen N of the sample was found to be 0·08 mg. equivalent to 0·211 ml. Therefore the I_2 uptake of the serum proteins = 1·72 ml./mg. N, which is a little lower than the value for fibrinogen (2·64 ml./mg. N).

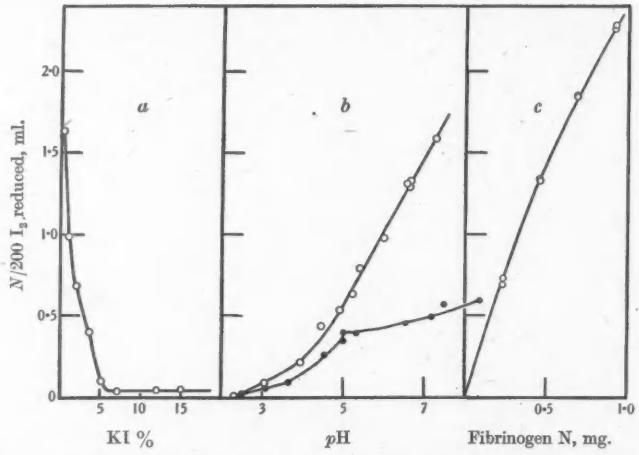

Fig. 1. Effect of various factors on the reaction: *a*, effect of potassium iodide; *b*, effect of *p*H; *c*, effect of fibrinogen concentration. o—o *N*/200 I_2 reduced; •—• *N*/200 H_2O_2 reduced. Fibrinogen present, as mg. N: *a*, 0·500; *b* (I_2), 0·425, *b* (H_2O_2), 0·850. Iodine or peroxide present, 1 ml. of *ca.* *N*/100. Total volume, 6 ml., and phosphate concentration *M*/24, in all.

Effect of denaturation on the reducing properties of fibrinogen

Mirsky & Anson [1936] found that denaturation of proteins increased their reduction of ferricyanide. This was not found with the I_2 reduction by fibrinogen (Table 2). The fibrinogen was denatured in a water-bath at 56°, when an increase in the reducing

Table 2

A. *Effect of denaturation on the reducing properties of fibrinogen*

Incubated at 56° min.	Insol. N mg.	$N/200$ I_2 reduced ml.	$N/200$ H_2O_2 reduced. ml.
0	0·000	1·167	0·372
0·5	0·022	1·174	—
2	0·252	1·195	0·366
5	0·325	1·194	0·355
10	—	1·143	0·322
36	0·322	1·094	—
60	—	1·038	0·200
240	—	0·847	—

(H_2O_2 consumption for 5 hr. and for 2 ml. fibrinogen solution (1·0 mg. fibrinogen N). I_2 uptake determined at 25° for 1 hr. for 1 ml. fibrinogen solution (0·5 mg. fibrinogen N).)

B. *Effect of denaturation on the reducing properties of plasma*

Temp. ° C.	$N/200$ I_2 consumed ml.
25	0·955
40	0·956
60	0·957
80	1·113
100	1·179

(Plasma diluted 1 : 25 with H_2O and exposed to the raised temperature for 5 min. I_2 consumption in 1 hr. at 25° was determined for 1 ml. of the diluted plasma.)

value of not more than 2 % occurs in the first 5 min. After prolonged heating, the reducing value falls rapidly, but this was due to a decrease in the rate of reaction, since no difference between the denatured and native fibrinogen was found when the I_2 uptake at equilibrium (12 hr. oxidation) was determined. Similar results were obtained when H_2O_2 was used, but there was no evidence of any increase in the reducing value, which shows a gradual but definite decrease from that of native fibrinogen. In view of the unstable nature of fibrinogen, it was of interest to see if atmospheric oxygen had any effect on the reducing properties of the protein. No difference between the reducing properties of samples of fibrinogen kept in N_2 or in O_2 could be detected, even after prolonged incubation at 56°.

The effect of alkali and acid, to which fibrinogen is very sensitive, was tested on the reducing properties of the protein. Samples of the protein were kept 24 hr. at 25° at various *p*H levels, and their I_2 consumption at *p*H 6·6 was then determined. There was very little effect, however, so that this lability is not associated with or accompanied by a change in the reducing groups.

While denaturation has little effect on the reducing properties of fibrinogen, it definitely increases the reducing power of plasma, as shown in Table 2B. 3 ml. of water were added to 1 ml. of a 1 : 25 dilution of plasma and the tube was placed in a water-bath at the desired temperature for 5 min. The tube was then cooled, 1 ml. phosphate buffer and 1 ml. I_2 added, and the I_2 consumption after 1 hr. determined. There is no increase in the I_2 consumption after heating at temperatures below 80°, i.e. the denaturation of fibrinogen causes no increase in the I_2 consumption. On heating to 100°, where denaturation of the serum proteins will occur, there is a definite increase (20 %) in the I_2 consumption.

Changes in the reducing power of fibrinogen during clotting

To 5 ml. solution (0·880 mg. fibrinogen N in $M/10$ phosphate buffer) was added 0·1 ml. of a crude thrombin solution (0·021 mg. N). After 10 min., when a firm clot had formed (fibrin N = 0·564 mg.), 1 ml. $N/75$ I_2 was added and the amount of I_2 reduced after 1 hr. determined. For the clot this was 1·18 ml., while for the fibrinogen and thrombin it was 2·24 + 0·065 = 2·30 ml. This result indicates less reduction by the fibrin but does not distinguish a slower rate of reaction from a lower reducing power. Therefore, the amounts of H_2O_2 and I_2 reduced by the time equilibrium had been attained (30 hr.) were determined. To 5 ml. solution containing 1·0 mg. fibrinogen N was added 0·1 ml. thrombin solution (0·01 mg. Parke, Davis thrombin). After 3 hr. (fibrin N = 0·762 mg.) 1 ml. $N/50$ H_2O_2 was added. 0·903 ml. $N/200$ H_2O_2 was reduced after clotting, compared with 0·819 ml. for the fibrinogen control. This represents an increase of 0·110 ml./mg. of fibrin N, i.e. 13 %. In a similar experiment with I_2, the amount of I_2 reduced after clotting (fibrin N = 0·362 mg., original fibrinogen N = 0·478 mg.) was 1·818 ml., that of the fibrinogen control, 1·812 ml.

The reducing power of fibrin at equilibrium was more accurately determined with commercial beef fibrin (Table 3). 10 mg. samples were weighed out on the micro-balance and 5 ml. $M/20$ phosphate buffer and 1 ml. H_2O_2 or I_2 solution added. The

Table 3. *The reduction of H_2O_2 and I_2 by fibrin*

Time hr.	$N/200$ H_2O_2 reduced ml.	Time hr.	$N/200$ I_2 reduced ml.
6	1·390	1·3	3·32
12	1·792	3	3·83
17	1·810	3·5	3·86
20	1·808	7·5	5·13
24	1·898	19	5·57
28	1·695	24	5·26
36	1·841	27	5·95
40	1·870	29	5·95

(10 mg. fibrin = 1·401 mg. N.)

average values for the amount of $N/200$ H_2O_2 and I_2 reduced at equilibrium were 1·823 and 5·57 ml. This gives values of 1·301 and 3·98 ml./mg. N. The previous values found for fibrinogen were 0·855 and 3·42 ml., so that the increase in reducing power is equivalent to 0·446 ml. H_2O_2 and 0·56 ml. I_2. The increase in reducing power is approximately the same with the two reagents but, owing to the more extensive oxidation by I_2, the increase with this reagent is a much smaller percentage of the total titration and hence the experimental error is increased accordingly. The increase is equivalent to half the reducing power of fibrinogen for H_2O_2.

These high concentrations of I_2 and H_2O_2 appear to have little effect on the activity of thrombin. Further, after oxidation of fibrinogen with H_2O_2, the addition of thrombin still results in a normal clot. The product resulting from the action of I_2 on the protein, however, is not clotted by thrombin, although thrombin still attacks it. The more extensive oxidation by I_2 evidently interferes with the gel properties of fibrin, since no clot is formed by the thrombin and the altered fibrin is merely precipitated.

The reduction of H_2O_2 by non-protein substances under the conditions tested

As a possible indication of the nature of the reducing groups of fibrinogen, 20 mg. of various substances were weighed into flasks, and 1 ml. of $M/4$ phosphate buffer and 4 ml. water added; 1 ml. of $N/75$ H_2O_2 was then added, the flask shaken for 28 hr., and the excess H_2O_2 titrated (Table 4).

Table 4. *Reduction of H_2O_2 by various substances*

Substance (20 mg. unless otherwise indicated)	$N/200$ H_2O_2 reduced ml.
l-Tyrosine	0·000
l-Tryptophan	0·246
2-Methyl-1:4-naphthoquinone	0·161
2-Methyl-1:4-naphthohydroquinone	0·247
Glucose	0·603
p-Dimethylamino-benzaldehyde	0·950
Casein	0·726
Fibrinogen	2·402
Cysteine HCl (1·0 mg.)	0·822
Ascorbic acid (0·1 mg.)	0·451

Tyrosine did not reduce H_2O_2. Tryptophan, glucose and vitamin K showed slight activity but this was very small compared with that of fibrinogen. Cysteine was more active than fibrinogen, but on the basis of these reduction figures fibrinogen would contain 15% cysteine and fibrin 22%: values reported for the cysteine content of fibrin are from 1·5 to 3·7% [Jordan Lloyd & Shore, 1938]. This suggests that cysteine is not responsible for the reduction by fibrinogen, and it seems reasonable to assume that the reducing group will not be more than 5% of the protein. The high reducing power of the active group is shown by the fact that only ascorbic acid gave a reduction of this order.

DISCUSSION

The characteristic reaction of fibrinogen with iodine appears to indicate a further example of the non-sulphydryl reducing groups of proteins studied by Mirsky & Anson [1936] and more recently by Bowman [1941 *a*, *b*] and ascribed by them to the tyrosine and tryptophan residues of the protein. Mirsky & Anson studied the reduction of ferricyanide by edestin, serum globulin, egg albumin and gelatin. There appear to be marked differences between the reaction studied by them and that reported here, although the same group appears to be involved. They found that their reaction was not clearly defined, for even when the ferricyanide concentration was 30 and 50 times that reduced by the protein, the amount reduced still depended on the ferricyanide concentration. This is in marked contrast to the present results, which show a clearly defined bimolecular reaction. Also, they found little reduction of the ferricyanide unless the protein had been denatured. Fibrinogen reduces no more H_2O_2 or I_2 after denaturation than before, although denatured serum proteins do show an increase in reduction. Bowman observed the reduction of I_2 by the chorionic gonadotropic hormone, and found that the reaction was inhibited by KI, and that the presence of phosphate markedly and specifically accelerates the reaction. The fibrinogen used in the above experiments was dissolved in phosphate buffer. Bowman found that in the presence of phosphate, tyrosine reduces I_2 very rapidly at room temperature, the reaction being influenced by KI and *p*H in the same way as the reduction by protein. But tryptophan may be equally responsible for the reducing power, since under the same conditions tryptophan shows equally strong reducing properties.

The results with H_2O_2 indicate, however, that fibrinogen contains another reducing group with much stronger reducing properties than tyrosine or tryptophan. This group reduces H_2O_2 at *p*H 5–7. Further, there is an apparent increase in the number of such groups in the protein on conversion of the fibrinogen to fibrin. The nature of this group is unknown, cysteine and ascorbic acid being the only simple substances tested which show similar reducing power; but the negative nitroprusside reaction and the quantitative relationships found suggest that the former is not responsible.

SUMMARY

The action of H_2O_2 and I_2 on fibrinogen has been studied. Fibrinogen in phosphate buffer solution reduces these substances rapidly at 25° and *p*H 6·6. The reaction with I_2 is associated with the aromatic residues of the protein. The reducing power of fibrinogen is not changed by denaturation, but the reducing power towards H_2O_2 is increased on conversion to fibrin.

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The Vitamin B_1 Content of Potatoes

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The use of the mould *Phycomyces blakesleeanus* as a test organism for estimating vitamin B_1 was first worked out by Schopfer & Jung [1937*b*]. The fungus responds to such small quantities of the vitamin that the method is the most sensitive known; on this account it was adapted for estimating the vitamin B_1 content of human blood by A. P. Meiklejohn [1937] and Sinclair [1938; 1939]. The method has been used to estimate vitamin B_1 in plant materials by several American workers [Burkholder & McVeigh, 1940; Robbins, 1939; Bonner & Erickson, 1938]. It has the advantage, as compared with other methods, of requiring such small samples of plant material that it can be used to study the distribution of the vitamin in a single plant or part of a plant.

This paper deals with the use of the *Phycomyces* method for estimating the vitamin B_1 content of potato tubers, and with variations in the vitamin content and its distribution in the potato plant.

METHODS

The method employed was an adaptation, for use with plant material, of the method for blood estimations worked out by Meiklejohn and Sinclair. It consisted essentially in the comparison of the growth of *Phycomyces* in cultures containing potato samples with a dose-response curve obtained from a series of control cultures containing known amounts of the vitamin.

Medium. The *Phycomyces* cultures were grown in 50 ml. conical flasks; each flask contained 4 ml. of a mixture of equal parts of the following solutions in distilled water:

Glucose (A.R.)	Equal weights of solid and water
Asparagine (recrystallized)	4 g./100 ml.
KH_2PO_4 (A.R.)	1·5 g./100 ml.
$MgSO_4, 7H_2O$ (A.R.)	0·5 g./100 ml.

The volume of liquid in each flask was made up to 10 ml. by the addition of vitamin B_1 solution and distilled water, or distilled water only.

Control cultures. A range of 12 control cultures was set up in every experiment, containing the following amounts of vitamin B_1 in 10 ml. liquid: none, 0·05, 0·1*, 0·15, 0·2*, 0·25, 0·3* μg., and excess (2·5 or 5 μg.) (quantities marked * in duplicate). A solution of vitamin B_1 containing 1 μg./ml. was freshly prepared for each experiment from a stock solution in 75% ethanol (*p*H 4·5) kept in a refrigerator. The required volume of the dilute solution was added to medium and water in each flask, immediately before the adjustment of the *p*H.

Potato samples. The size of sample was designed to give a reading in the central part of the dose-response curve. Preliminary experiments showed that, for potato, samples of between 0·1 and 0·2 g. were correct. According to the amount of vitamin expected, samples were taken of 0·1, 0·15, or 0·2 g. (±10 mg.), in triplicate from each tuber, or part of a tuber, to be tested, by taking out cores with a small cork borer and cutting off the required length. Skin samples were taken by removing the corky skin and cutting a thin slice off the exposed surface, and sprout and leaf samples by cutting off a small piece. All samples were

quickly weighed and immediately blanched by putting them straight into the liquid medium in the culture flasks, which were held at a temperature of 80–100° in a steamer.

Sterilization. All culture flasks were sterilized by steaming for 45 min. on 3 successive days. Just before the second steaming, the dilute vitamin B_1 solution was added to the control flasks; some of the potato cultures received excess vitamin as well, and this was added at the same time, the *p*H of the liquid in all flasks being brought to 6·4 with *N*/20 NaOH. The potato samples were broken up with a glass rod.

Inoculation and incubation. Stock cultures of *P. blakesleeanus* (sex −) were maintained on dextrose-peptone-malt agar, and also on the experimental liquid medium, with potato and excess vitamin B_1. Inocula were made, normally from a liquid culture, by removing the aerial mycelium and stirring it in sterile distilled water. 1 ml. of the resulting spore suspension was pipetted into each culture flask. The flasks were incubated 12–16 days in the dark at room temperature.

Drying and weighing of fungus. At the end of incubation the mats of *Phycomyces* mycelium were removed from the culture flasks, washed 3 times in tap and once in distilled water, dried for 2–3 hr. at 100° and weighed to 0·1 mg.

Calculation of results

A dose-response curve was constructed from the 12 control cultures in every experiment by plotting the dry weight of mycelium in mg. (or the average of duplicates) against the logarithm of the amount of vitamin B_1 (μg.) in each culture. The curves were always S-shaped, and the middle part, corresponding to 0·1–0·25 μg. vitamin B_1, was very nearly a straight line [cf. Sinclair, 1939]. The amount of vitamin in the potato samples was calculated by reading from the curve the figure corresponding to the dry weight of fungus from each sample flask. The results are expressed as μg. vitamin B_1/100 g. fresh weight of potato.

RESULTS

A. *Comparison with thiochrome method.* The *Phycomyces* method is open to one serious theoretical objection; Schopfer & Jung [1937*a*] showed that the fungus responds to the pyrimidine and thiazole halves of vitamin B_1, if they are supplied together, as well as to the intact vitamin. It was therefore thought advisable to check the fungus method by comparing it with the thiochrome method as described by Pyke [1939]. The results of 3 comparisons on tubers of the variety Majestic are given in Table 1; the figures obtained by the 2 methods are of the same order in each case. This makes it reasonably certain that vitamin B_1 itself, and not its decomposition products, was being estimated by the fungus method.

B. *Variations during growing season.* In both 1941 and 1942, at intervals during the growing season, between July and late September, tubers were dug from plants in the same field and assayed for vitamin B_1. In 1941 the material was obtained from Spalding, Lincolnshire, by Mr P. H. Gregory; the two varieties Majestic and King Edward, growing in adjacent fields, were sampled by taking 1 tuber from each of 4 plants of each variety for each determination. In 1942 the material was obtained from a plot on the Rothamsted farm planted with Scottish seed Majestic; 2 tubers were taken from each of 2 plants at each sampling. Three cores were taken from the middle of each tuber.

From Table 2 it will be seen, first, that the samples are reasonably constant in vitamin B_1 content; the standard errors of the mean values for each sample are small enough to permit a valid comparison between samples.

Secondly, the vitamin B_1 content of the tubers of both varieties increases continuously during the growing season. The figures for Majestic show that the increase continues up to the time when maincrop potatoes are usually lifted, and also show a very good agreement between parallel samples in

Table 1. *Comparison of methods for estimation of vitamin B_1*

Date	Nature of material	Comparison	Vitamin B_1 (μg./100 g.) Fungus method	Vitamin B_1 (μg./100 g.) Thiochrome
April 1942	Stored tubers	Different tubers of same batch	76	61
			76	72
May 1942	Seed tubers (sprouted)	Different tubers of same batch	57	54
			46	—
			78	—
July 1942	Freshly dug young tuber	Both tests on same tuber	51	67

Table 2. *Variations in vitamin B_1 content of tubers dug in summer*

Date of digging	Majestic 1941 (μg./100 g.)	King Edward 1941 (μg./100 g.)	Date of digging	Majestic 1942 (μg./100 g.)
18. vii. 41	61 ±1·6	63·1 ±4·0	20. vii. 42	55·6 ± 4·7
9. viii. 41	74·8 ±3·9	67 ±2·1	12. viii. 42	75·6 ± 5·1
8. ix. 41	91·3 ±4·6	77·3 ±7·4	15. ix. 42	135·7 ±10·2
25. ix. 42	141·3 ±8·7			

Each figure is the mean of 12 determinations: 3 cores each from 4 tubers.

Table 3. *Effect of storage and sprouting on vitamin B_1 content of potatoes*

Date	Where stored	Variety	No. of tubers	Vitamin B_1 (μg./100 g.)	Variety	No. of tubers	Vitamin B_1 (μg./100 g.)
25. ix. 41	Crop as lifted	Majestic	4	141·3 ± 8·7	Doon Star	4	163 ±12·5
2. i. 42	Rothamsted	,,	3	162·2 ±13·0	—	—	—
	Sutton Bonington		3	138·2 ±12·3	—	—	—
5. ii. 42	Rothamsted	Majestic (sprouted)	4	112·4 ±22·2	King Edward (sprouted)	4	46·7 ± 7·8
8. iv. 42	Sutton Bonington	,,	4	99·9 ± 8·5	,,	4	73·7 ± 4·4
7. v. 42	Scottish seed fully sprouted	,,	3	61·8 ± 7·0	—	—	

1941 and 1942. King Edward tubers would appear to have a lower vitamin B_1 content than Majestic tubers.

C. *Effect of storage.* During the winter and spring of 1941–2, samples were taken from tubers stored in two places: at Rothamsted, where they were kept in paper bags on the laboratory floor, and at the Midland Agricultural College, Sutton Bonington, on trays in a well-ventilated storehouse. The cores for vitamin determinations (3 or 4) were again taken from the centre of each tuber (Table 3).

There certainly is a loss of vitamin B_1 from the centre of the tuber during storage, and apparently the greatest loss takes place in spring, after the tubers have begun to sprout. It may be conjectured that the greater part of the loss is due to sprouting, and not to storage *per se*. This conjecture is supported by the gradient in sprouting tubers (see Section D). Unfortunately there are not many results from stored material, and the material in some cases varied considerably in vitamin B_1 content. This is particularly so in the case of the Rothamsted-stored Majestic, as is shown by the high standard error.

D. *Distribution in the plants. Gradients.* It was thought possible that there might be a movement of vitamin B_1 into the rose end of a sprouting tuber, which would result in a gradient in vitamin content within the tuber. At different times of year, therefore, tubers were cut into 3 parts: the rose end, a middle slice (which is the part usually taken for sampling), and the heel end. Cores were taken from the 3 parts separately for determinations (Table 4).

There is consistently less vitamin in the heel end than in the rest of the tuber, but the evidence for a gradient cannot be regarded as satisfactory. There is, however, a very much higher content in sprouts than in the middle of the tuber, as is shown in Table 5. (These figures were obtained from white sprouts only, as green sprouts showed a curious anomaly, which is discussed in a later section.)

Table 4. *Distribution of vitamin B_1 within single tubers*

Date	Variety	Condition of tuber	Position	Vitamin B_1 (μg./100 g.)
May	Majestic	Fully sprouted seed	Heel	38·4
			Middle	77·8
			Rose	67·7
Oct.	Majestic	Freshly dug, no sprouts	Heel	97·9
			Middle	101·7
			Rose	120·1
Oct.	Majestic	Freshly dug, no sprouts	Heel	117·8
			Middle	209·9
			Rose	165·7

Table 5. *Sprouts compared with centre of tuber*

Date	Variety	Vitamin B_1 in sprouts (μg./100 g.)	Vitamin B_1 in tuber (μg./100 g.)
Jan.	Majestic	174	128
Feb.	Majestic	149	20
Feb.	King Edward	250	53

There was always less vitamin in the layer immediately under the corky skin than in the centre of the tuber; comparisons on different single tubers are given in Table 6. The skin layer, however, was always found to show adjuvant action; this is further discussed in a later section of this paper.

Table 6. *Skin compared with centre of tuber*

Date	Variety	Vitamin B_1 in centre (μg./100 g.)	Vitamin B_1 in skin layer (μg./100 g.)
Jan.	Majestic	179	22
Feb.	Majestic	206	9
		76	10
Feb.	King Edward	24	21
		49	18
May	Majestic	78	33

There is a very marked gradient of vitamin content in growing potato plants; in June 1942 two seed tubers of Majestic which had been planted a month previously were dug up and assayed together

with the small plants growing from them, which were then about 4 in. above the ground. Samples were taken from the heel, middle and rose ends of the seed tubers, the white stem below ground, and from the small leaves at the top of the stem. Sampling from 2 whole plants was repeated in July, when the tops were about 18 in. high. In this case samples were taken from the middle of 2 newly developed tubers, from an internode half-way up the stem, from the blades of a lower leaf, and from the terminal bud and the small leaves round it. The averages of 3 determinations at each point are given in Table 7.

Table 7. *Gradients in vitamin B_1 content in growing plants*

Date	Part of plant	Vitamin B_1 (μg./100 g.) Plant A	Plant B
8 June	Planted seed tuber: heel	25	27
	middle	39	24
	rose	47	40
	White stem below ground	103	93
	Foliage—small leaves	>1200	>1200
		Plant C	Plant D
20 July	Young tubers—middle	53	51
		78	41
	Green stem—internode	9	9
	Lower leaf—blade	129	153
	Terminal bud and leaves round it	>285	>115

In June the planted seed tubers have their highest vitamin B_1 content in the rose end; the stem has a rather larger content, and the value for the tops is enormously high. The amount of mycelial growth in the cultures on foliage was so large as to be right off the curve; the true vitamin content can only be guessed at. In July stems contain very little vitamin and leaves a good deal; but even the terminal buds do not approach the enormously high figure for June foliage.

E. *The presence in potato of adjuvant factors for the growth of* Phycomyces. *Phycomyces* responds to other growth factors than vitamin B_1; Schopfer & Jung [1937*b*] said that it requires 'une constellation de facteurs auxogènes.' Sinclair [1938] described the 'adjuvant action' of blood; blood and vitamin B_1 supplied to the fungus together produced more growth than the sum of the two separately. He attributes this action to a combination of causes, notably the buffering action exerted by blood, and its nitrogenous constituents.

In every experiment reported in this paper some cultures were grown on potato and excess vitamin, to test for the presence of adjuvant action; such action, shown by excess growth over the sum of potato and vitamin cultures separately, was exhibited by tuber material only in the cases shown in Table 8.

Table 8. *Potato tubers showing adjuvant action in vitamin B_1 test*

Date	Variety	Condition of tuber
1941		
June	Majestic	Fully sprouted seed
July	Majestic	Freshly dug, newly formed
Aug.	Majestic	Freshly dug, newly formed
1942		
Apr.	King Edward	From store, sprouted
June	Majestic	Seed, 1 month planted
July	Majestic	Freshly dug, newly formed
Aug.	Majestic	Freshly dug, newly formed

Mature tubers, from September to April, never displayed any adjuvant action, nor did seed potatoes at the time of planting in 1942. Samples of skin layer (see Table 6) always showed a marked action, but the factor was not present in sprouts. Stem from growing plants (Table 7) showed adjuvant action in 2 cases, but leaves never did.

The adjuvant factor in potato apparently operated only in presence of excess vitamin B_1, as is shown by the experiment reported in Table 9, in which parallel sets of cultures were set up, 1 with a range of concentrations of vitamin B_1 alone (column A), a 2nd with the same range of vitamin concentrations plus potato samples taken from a single tuber (column B), and a 3rd set containing samples from the same tuber without added vitamin (column C). The increased growth due to the addition of potato (B − A) is no larger than that produced by potato alone; except in the presence of excess vitamin (2·5 mg.).

Table 9. *Growth of fungus with potato alone and on potato plus vitamin B_1*

Vitamin B_1 supplied (μg.)	Fungus growth with vitamin B_1 only (mg.) A	Fungus growth with vitamin B_1 + potato (mg.) B	Diff. (B − A)	Growth with potato alone (mg.) C	Adjuvant action
0·05	18·2	51·7	33·5	36·8	None
0·1	31·2	72·2	41·0	42·0	None
0·2	57·2	87·8	30·6	39·2	None
0·3	69·2	103·2	34·0	36·8	None
2·5	89·2	155·6	66·4	—	+

As the adjuvant factor only appears to operate after the need of the fungus for vitamin B_1 has been satisfied, it was not allowed for in calculating the results [cf. Sinclair, 1939], as it was assumed that the vitamin supply was acting as a limiting factor under the conditions of the experiment, so that the growth of the fungus was in fact a measure of the vitamin present.

The adjuvant action of potato does not appear to be due to the same causes as that of blood; it is

not due to buffering; cultures with and without potato both reach a *p*H value of 4·0 at the end of incubation. Neither is it due to nitrogenous compounds; in the absence of asparagine, potato and excess vitamin give very little growth as compared with blood and excess vitamin. This is seen in Table 10, which also shows the additive effect of potato and blood when supplied together; this is further evidence that the factors in potato and blood are distinct.

Table 10. *Growth of mycelium on blood and on potato*

	Additions to medium			Growth of mycelium	
Vitamin B_1 (μg.)	Potato (g.)	Blood (ml.)	Asparagine (mg.)	(mg.)	Excess growth in presence of both factors (mg.)
5	—	—	40	77	—
—	0·15	—	40	38	—
—	—	1	40	29	—
5	0·15	—	40	121	6
5	—	1	40	136	30
5	0·15	1	40	188	44
5	0·15	—	—	12	—
5	—	1	—	115	—

It is possible that the adjuvant action of potato observed in these experiments is due to the 'factor Z' described by Robbins [1939]. It is interesting in this connexion that royal jelly from a queen bee cell, which has been reported to be rich in pantothenic acid [Pearson & Burgin, 1941] had no stimulating action whatever upon *P. blakesleeanus*, either in the presence or absence of excess vitamin B_1.

F. *The anomalous action of green sprouts.* White sprouts from potatoes kept in the dark (Table 5) were found to have a high vitamin content. All attempts to repeat this work on green sprouts, however, failed completely. In presence of green sprouts the fungus did not grow at all; there was no visible mycelium, and the *p*H of the medium was unchanged. A second inoculation of fungus spores into the same flask failed like the first. It seems likely, to judge from the high vitamin B_1 content of white sprouts and young leaves, that green sprouts also have a high content, but this cannot be proved, because of their toxic action on the fungus.

It was thought that the toxic action might be due to the solanine in the sprouts; accordingly solanine and solanidine were supplied to the fungus under a variety of conditions, but with very little effect. 2 mg. solanine produced a slight diminution of growth, but smaller quantities no effect at all; the same was true of 4 mg. and smaller quantities of solanidine. There was only one exception: in a single case complete inhibition of growth occurred in a culture containing white sprouts and 2 mg. solanine. It cannot be pretended that the toxic action of green sprouts is explained, but it may be noted that the thiochrome method also failed on green sprouts; the pepsin digest, which was coffee-coloured, gave no thiochrome reaction at all.

DISCUSSION

The vitamin B_1 content of potato tubers is important from the point of view of nutrition, as sources of this vitamin are somewhat restricted in a war-time diet. It appears from this work that mature tubers of the variety Majestic have a vitamin B_1 content, in autumn, of about 140 μg./100 g. On this basis a daily ration of 12 oz. of potatoes, if properly cooked in their skins, would supply 470 μg. of vitamin, or nearly half the usual estimate of the daily requirement of the vitamin (1 mg.). Potatoes, which are eaten by the great majority of people, are at any rate not a negligible source of the vitamin, particularly in the autumn and winter months.

The vitamin B_1 content of the young tubers increases continuously during the growing season; from this fact, and from the very high vitamin content of potato leaves, it can be presumed that the vitamin is synthesized in the leaves in summer and transferred to the young tubers as they grow. In winter the vitamin apparently remains in the tuber till sprouting begins, when it moves out of the centre of the tuber into the sprouts; but further evidence is needed on this point.

It is interesting to note that, in contrast to ascorbic acid, which is usually concentrated in the skin and adjacent layers of fruits and vegetables, vitamin B_1 in potatoes is concentrated in the centre of the tuber rather than in the skin.

The adjuvant action, which is due to some factor or combination of factors not fully explained, appears in tubers only in certain definite physiological states, e.g. in old fully sprouted, and young developing, tubers, between April and August. Adjuvant factors in potato have previously been described [Robbins, 1939], but information is lacking as to the age or physiological state of the tubers from which they were extracted. It can definitely be said that the adjuvant action of potato is due to a different cause from that of blood, but the factor responsible cannot be identified with any previously described.

SUMMARY

1. The *Phycomyces* method of estimation is valid for determining the amount of vitamin B_1 in potatoes.

2. The vitamin B_1 content of Majestic tubers at the time of lifting (September) is about 140 μg./100 g.

3. During the growing season the vitamin B_1 content of tubers increases continuously up to the time of lifting of maincrop potatoes.

4. The vitamin B_1 content of stored tubers falls during the winter and spring; the greater part of the loss takes place in spring, and is apparently due rather to sprouting than to storage, especially as white sprouts have a higher vitamin B_1 content than the tuber which produces them.

5. The vitamin B_1 content of young potato leaves is very high.

6. An adjuvant factor of undetermined nature is present in the centres of tubers between April and August, and always present in the skin layer.

7. Green sprouts completely inhibit the growth of the fungus.

I should like to express my thanks to the following: to F. C. Bawden, under whose auspices the work was carried out, and N. W. Pirie, for advice; to P. H. Gregory for potato samples and help in planning the experiments; to H. M. Sinclair for a culture of *Phycomyces blakesleeanus*; to my brother, A. P. Meiklejohn, for calling my attention to the possibilities of the method, and to E. M. Crook and D. J. Higgons for supplies of solanine and solanidine.

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The Total Nitrogen Content of Egg Albumin and Other Proteins

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The question of amino-acid analysis became one of increasing importance in this laboratory during 1933, when the isolation of proteins from various forage grasses had been successfully accomplished and evidence for their content of the dietetically essential amino-acids was clearly desirable.

Work has continued without interruption since then, but at times progress has been slow on account of the laborious nature of many of the incidental investigations that we have felt called upon to undertake. During the first two years some preliminary studies of the better-known methods for base and dicarboxylic acid analyses were made by Miller [1935; 1936]; these served to emphasize the large amount of humin formation that attended the hydrolysis of our relatively impure leaf products, and little further progress was made until the origin of this humin had been satisfactorily established by Bailey [1937] and Lugg [1939]. The observation that cystine and, to a lesser extent, tyrosine can condense, like tryptophan, with the furfural derived from pentosan-like impurities during acid hydrolysis to form humin led Lugg, in continuation of work started in Adelaide, to investigate the reliability and limitations of the better-known indirect methods for estimating these three amino-acids, and also methionine [cf. Lugg, 1939]. Meanwhile, Tristram [1939] had explored the applicability to these impure leaf proteins of the Block small-scale gravimetric method of base analysis; progress had been made in an attempt to improve the Foreman method of dicarboxylic acid analysis; and the separation of the monoamino-acids by the Dakin-Fischer methods had been explored. With the experience thus gained it was possible to carry out partial amino-acid analyses of a series of protein preparations from forage crops [cf. Chibnall, 1939].

At this stage in our work we were not specifically interested in obtaining strictly quantitative values for the dicarboxylic acids and bases, for such a refinement was considered unnecessary in view of the somewhat impure nature of our leaf products; all that we had set out to attain was an analysis for each product comparable, from a nutritive point of view, with those already in existence for many seed and animal proteins. During 1936–8 however Bergmann & Niemann reported partial analyses of several proteins, and on the results of these they formulated certain stoichiometric laws governing protein structure. Two of the proteins they had selected for consideration, egg albumin and haemoglobin, had already been submitted to dicarboxylic acid analysis in this laboratory, and our results, which we were fairly certain were still several per cent too low, were higher than theirs and definitely not in keeping with the general conclusions they had drawn. Moreover, in agreement we believe with many others, we were not satisfied with the values they had assigned to the molecular weight of the average amino-acid residue.

In 1938 therefore it was decided to attempt to perfect our analytical methods for the dicarboxylic acids and the bases so as to obtain values which we could feel were reliable to 2% or less. In this we have been as successful as we could reasonably expect, and our investigations, which are still in progress, will be reported in later papers. Meanwhile the bearing that these new results have on the structure of the proteins concerned has been discussed in a recent lecture [Chibnall, 1942], and the interpretation was based in part on a value for the molecular weight of the average amino-acid residue computed from data which include, *inter alia*, the total N of the protein. As it is essential that this latter value be accurately determined we were led to the investigations reported in the present communication.

On reviewing the more recent literature dealing with the preparation and analysis of proteins we found that in many instances the recorded value for the total N of the protein concerned was significantly lower than that accepted by earlier reputable analysts such as Osborne and Sörensen, and the disagreement was especially marked in the case of egg albumin. Now it is common knowledge that the two above-mentioned workers used methods of analysis which they had proved by experience were reliable, and the implication that the work of the later investigators might be untrustworthy needed demonstration, for the position left us not only in doubt as to the value to be used in the computation of the average residue weight, but, as will be discussed later, it shook our confidence in the results of many of the more recent amino-acid analyses of protein hydrolysates and physicochemical studies of proteins in solution.

Osborne invariably determined N on the air-dried sample, as he found that, in general, the anhydrous material was too hygroscopic to be handled without special precautions. For the Kjeldahl analysis he used about 0·1 g. protein, 20 ml. H_2SO_4 and a drop of mercury, the heating being continued for 4–5 hr. after clearing and the oxidation completed by adding a few small crystals of $KMnO_4$ immediately after the gas to the burner had been turned off. Sörensen & Höyrup [1915–17] took for analysis an amount of egg albumin solution corresponding to not more than 20 mg. protein-N and digested this with 20 ml. H_2SO_4, 5 g. K_2SO_4 and 50 mg. Cu wire. The digest cleared in 2–4 hr. and the heating was continued for a further 5 hr. The still boiling liquid was then oxidized by adding cautiously 1·5 g. of dry pulverized $KMnO_4$ in 3 equal lots. Both these protein chemists therefore found from experience that prolonged heating of the digest was necessary for an accurate analysis.

In recent years there has been a natural desire to shorten the time period of digestion and this has led to the introduction of new catalysts such as selenium, its dioxide, or metallic selenates, all of which have been shown to be very effective in promoting the rapid oxidation of many forms of organic-N [cf. Bradstreet, 1940]. These modifications, often applied on a micro-scale, have been shown in many instances to give a satisfactory recovery of N if the heating be continued for less than 1 hr. after clearing, and the notion that the time lag inherent in the older methods had been thereby overcome has no doubt been fostered by the undoubted success that has attended most of the rapid micro-methods of organic analysis introduced by Pregl. When these modified Kjeldahl methods are applied, however, to many materials of biological origin (e.g. wheat, flour, seeds, etc.) there is no absolute check on the results obtained, and in the protein and amino-acid field the uncritical acceptance of their trustworthiness is apparent from the data collected in Table 1 and the discussion which follows.

Table 1. *Nitrogen content of certain proteins, as recorded in the literature*

(All values are calculated on an ash-free and moisture-free basis; those in parentheses indicate the ranges with different samples. K indicates Kjeldahl analysis; D, Dumas analysis.)

Protein	Total N (%)	Reference
Egg albumin, heat coagulated	K 15·55 (15·51–15·59) D 15·6 (15·57–15·63)	Osborne & Campbell [1900]
Egg albumin, heat coagulated	K 15·71 (15·61–15·83)	Vickery & Shore [1932]
Egg albumin, native protein	K 15·60 (15·61–15·59)	Taylor, Adair & Adair [1932]
Egg albumin, heat coagulated	K 15·16	Jones & Moeller [1928]
Egg albumin, heat coagulated	K 15·12 (15·03–15·23)	Calvery [1932]
Egg albumin, native protein	K 15·44 (15·1–15·7)*	Hendrix & Dennis [1938]
Egg albumin, native protein	K 15·36 (15·2–15·6)*	Hendrix & Dennis [1938]
Egg albumin, heat denatured	K 15·27	Bernhardt [1940]
Pepsin, twice recrystallized	K 13·95 (13·8–14·1) D 14·8 (14·63–14·83)	Northrop [1939]
Pepsin	K 15·4	Calvery, Block & Shock [1936]
β-Lactoglobulin, recrystallized	K 15·3†	Palmer [1934]
β-Lactoglobulin, 4 times recrystallized	K 15·14, 15·26‡	Pedersen [1936]
β-Lactoglobulin, thrice recrystallized	? 14·35	Winnick [1942]
β-Lactoglobulin, thrice recrystallized	K 15·6	Cannan [private communication]
β-Lactoglobulin	K 15·6	Sörensen & Palmer [1938]

* Ranges of repeat analyses of 1 sample. † Arnold Gunning method. ‡ Gunning-Pregl micro-method.

Osborne & Campbell [1900] made 4 preparations of what they considered to be pure coagulated egg albumin and obtained concordant values for total N by both the Kjeldahl and Dumas methods (Table 1). Sörensen & Höyrup [1915–17] discussed the precautions necessary to obtain a maximum value by the Kjeldahl method for the protein-N present in an $(NH_4)_2SO_4$ solution of the recrystallized protein, and concluded (p. 181) that 'The factor by which the weight of protein nitrogen has to be multiplied to give the weight of the egg albumin dried over solid potassium hydroxide *in vacuo* at indoor temperature is nearly equal to 6·4 and this does not apply solely to soluble crystallizable egg albumin, but also to denatured egg albumin and eventual middle terms between these two substances'. A factor of 6·4 corresponds to 15·63% N. More recently Vickery & Shore [1932] have obtained a mean value of 15·71% N for 3 samples of thrice recrystallized protein, while Taylor, Adair & Adair [1932] record a mean value of 15·60% N for 2 samples of protein that had been crystallized 7 times. In contrast to these high values, which show reasonable agreement, there are others, much lower, which exhibit considerable irregularities. All these latter appear to have been obtained from samples of recrystallized egg albumin, or of heat-coagulated products derived from them; hence one is led to infer that either the analytical procedures employed must have been faulty or the workers concerned have not realized that the anhydrous products are extremely hygroscopic. Calvery [1932] analysed many preparations of the coagulated protein and recorded a mean value of 15·12% (Table 1). Rather surprisingly this result is referred to later [Calvery, Block & Shock, 1936] in the following terms: 'It has been previously definitely established that the nitrogen content of crystalline egg albumin when dried at 103–110° and corrected for ash is 15·4%.' Hendrix & Dennis [1938] made a very large number of analyses of 2 samples of crystalline protein, and also of denatured products obtained from them by the action of dilute alkali, dilute acid and by shaking. Sörensen & Höyrup's technique was employed, and the products were dried to constant weight *in vacuo* at 110° before analysis. The results in each case exhibited considerable irregularities and were submitted to statistical treatment. The values for the native proteins are given in Table 1; those for the alkali-denatured (2 samples), acid-denatured and shaking-denatured products, were 15·21, 15·05, 14·86 and 14·95% N respectively. Robinson & Hogden [1941] determined the percentage N in 66 samples of heat-coagulated proteins from blood serum by a micro-method using $SeOCl_2$ as a catalyst, the heating being continued for only 20 min. after clearing. Their analyses were made on material dried to constant weight, and the difficulty of dealing with this on account of its hygroscopic nature was admitted; the results ranged from 15·4 to 16·2% with a mean of 15·78% N. No control analysis of a standard protein of known N content was recorded. The above citations are by no means exhaustive. A search of the literature covering the last 15 years, the period during which the micro-Kjeldahl method has come more and more into general use, reveals many instances in which investigators have recorded the results of amino-acid analyses in terms of percentage weight of protein, the values being admittedly based on a determination of total hydrolysate-N. In these cases it is rare to find any mention of the mode of preparation or of the purity of the protein used; all too often its N content is not recorded and no indication is given of the procedure by means of which the hydrolysate-N was determined, except perhaps a simple statement that the micro-Kjeldahl method had been employed. The results given below illustrate the magnitude of the errors which may be incurred if insufficient attention be given to what is one of the simplest of analytical procedures.

EXPERIMENTAL

Protein samples analysed

Egg albumin. We have to thank Prof. R. K. Cannan for 2 samples of thrice recrystallized material [Kekwick & Cannan, 1936]. The coagulated protein was prepared in the following way. 160 g. of air-dried material, containing 26·5% Na_2SO_4, were dissolved in 1·5 l. H_2O and poured slowly, with vigorous stirring, into 15 l. H_2O at 90°. The coagulated protein was filtered off on a Büchner funnel, the mass removed from the paper, stirred up with 15 l. H_2O at 90° and again filtered. The washing was repeated, first with 15 l. H_2O at 90° and then successively with 2–3 l. cold H_2O until the last few filtrates were free from sulphate. The product was next washed with ethanol and ether, dried at 70°, ground to a fine powder and passed through a small-meshed sieve. The final product sampled reproducibly for moisture after storage in a bottle at room temperature for 2 years.

Edestin. This was prepared from freshly ground hemp seed in the usual way and twice recrystallized from hot 5% NaCl solution [Bailey, 1942].

Amandin. This globulin is of interest as it gives on hydrolysis an unusually high percentage of amide-N. Osborne & Campbell [1896] record a mean value of about 19·3% N by the Dumas method, but Osborne [1910] in a later review recalled that earlier workers such as Ritthausen found only about 18·7% and gave it as his opinion that 19·3% was probably a little too high and suggested 19·0%. We have to thank Dr Vickery for a sample of this protein prepared from almond seeds in 1915 by Mr Leavenworth. It contained, ash- and moisture-free, 18·66% N. The product, as was to be expected after the lapse of so many years, left a considerable insoluble residue when extracted with salt solution, and as we were anxious to obtain good crystalline material for various purposes purification was effected in the following way on the advice of Dr K. Bailey.

50 g. protein, 6·25 g. NaCl, 125 ml. H_2O and 6·25 ml. ethanol were ground to a paste in a mortar. *N*/14 NaOH was then added with stirring until the mixture was just blue to thymol blue (glass electrode *p*H 7·8–8·2). The volume was next adjusted to 1250 ml. with H_2O, the mixture brought to 25° and shaken gently without frothing for ½ hr., care being taken to ensure that all sticky particles had been dispersed. The mixture was then filtered 3 times through pulp that had been washed with distilled water. The filtrate was clear, and some insoluble dark brown material was left on the pad. *N*/10 HCl was added to the filtrate with rapid stirring until there was a definite sign of turbidity, and the solution then carefully adjusted to *p*H 6·3. At this stage the precipitate was not bulky and consisted of denatured protein, as shown by adding NaCl to a test sample and gently warming. The mixture was filtered on a well-washed pad, and to the clear filtrate was added with stirring 1/10 vol. of 95% ethanol. A white bulky precipitate appeared, which redissolved on warming to 35°. The clear solution was now cooled rapidly with stirring to 0°, left for 1 hr. at that temperature and the separated crystalline material centrifuged off as rapidly as possible in cooled cups. The paste thus obtained was stirred up with about 3 times its volume of 10% acetone, cooled to 0° and again centrifuged. The product was washed twice with 50% ethanol, 3 times with absolute ethanol, and then 3 times with acetone, being filtered off the last time and sucked free from all extraneous solvent. The final product was dried *in vacuo*. The yield was 18 g.

Haemoglobin. We have to thank Prof. E. J. Cohn for a sample of horse carboxyhaemoglobin prepared in November 1938 by Singher and Morro of the Harvard Medical School. It had been thrice recrystallized, heat coagulated and washed free from chloride. As received the product contained a small amount of fibrous material, undoubtedly filter pulp, which was removed by sifting.

β-Lactoglobulin. We have to thank Prof. R. K. Cannan for two 50 g. samples. The twice recrystallized protein had been coagulated by heat, washed free from inorganic salt and dried through ethanol and ether.

Excelsin. We have to thank Dr K. Bailey for a sample of the recrystallized protein [Astbury, Dickinson & Bailey, 1935].

Casein. This was prepared by the method of Cohn & Hendry [1923], except that the solution was clarified by filtration through paper pulp instead of being passed through a Sharples centrifuge.

Insulin. A crystalline sample was obtained from Boots Pure Drug Company Ltd., Nottingham.

Pepsin. We have to thank Dr J. St L. Philpot for 2 small samples of crystalline material.

Methods of analysis

Moisture and ash contents of proteins. The material for analysis was first finely ground in an agate mortar. For the determination of the moisture content a sample was dried in an oven at 102° to constant weight [Brand & Kassell, 1942]. In general this was attained in 3–5 hr. and the further loss on heating overnight did not exceed 0·1%. The ash content was determined by heating a sample of the air-dried material in a muffle at 550–600° to constant weight.

Kjeldahl incineration. Na selenate was first used as a Kjeldahl catalyst in this laboratory in 1935, and our notebooks show that prior to the present investigation, which was started in 1938, we had made use of a digestion mixture consisting of 20 ml. H_2SO_4, 10 g. K_2SO_4 and 25 mg. Na selenate. In addition 0·65 g. $CuSO_4.5H_2O$ was occasionally added. The recorded results for good recrystallized edestin were 18·4 ± 0·1% N, and for coagulated egg albumin 15·3 ± 0·1% N. When we decided in 1938 to make an amino-acid analysis of these two proteins the difference between our values and those quoted by Osborne came into sharp focus and we realized that our analytical methods needed overhaul. The trouble was finally traced to the short period of digestion (2–3 hr. after clearing) we had been employing and the following procedure was found, after a long trial of many, including those of Osborne, Sörensen, Pregl ($HgSO_4$), and Peters & Van Slyke [1932], to give the most satisfactory results with proteins and protein hydrolysates.

Our experience confirms that of Osborne and many others that oven-dried proteins are too hygroscopic for convenient handling, and we have invariably used air-dried material if sufficient were available for a separate determination of the moisture content. The sample for analysis, which should not contain more than 10–15 mg. N, is weighed out in a small glass flat-bottomed cup, stoppered if there be need to dry to constant weight. After weighing, the cup is dropped carefully into a long-necked 250 ml. Kjeldahl flask in such a way that no protein is scattered; about 5 ml. H_2O followed by 20 ml. N-free H_2SO_4 are added and the mixture boiled gently on the stand until charring commences. The flask is then removed from the stand, cooled slightly and 5 g. catalyst mixture added. The latter is prepared by grinding to a fine powder 80 g. K_2SO_4, 20 g. $CuSO_4.5H_2O$ and 0·34 g. Na selenate. The flask is then returned to the stand and the contents gently boiled. The digest clears in 15–30 min. and the heating is continued fairly vigorously for another 8 hr. or, if convenient, overnight. The cooled digest is finally made up to standard volume and an aliquot withdrawn for NH_3 estimation. It is essential, of course, that control blanks for the complete procedure be carried out at frequent intervals. In the case of protein hydrolysates an aliquot containing about 0·1 mg. N is withdrawn and incinerated in the usual micro-Kjeldahl tube, using 2 ml. acid and 0·5 g. catalyst mixture. The procedure then follows that given above, except that the whole digest is used for the NH_3 estimation.

Van Slyke, Hiller & Dillon [1942] have recently drawn attention to the difficulty of determining the N content of lysine by Kjeldahl analysis. According to our experience low values will always be obtained with this amino-acid unless the digest is boiled much more vigorously than is necessary when dealing with a protein rich in lysine such as lactoglobulin. Using a sample of *dl*-lysine monohydrochloride which gave 15·28% amino-N in 30 min. we have obtained a theoretical recovery of N under our usual conditions provided that the digest is boiled briskly on an electric heater for about 12 hr. Using a gas burner the recovery is irregular (95–100%) and it is advisable to add $HgCl_2$ to the catalyst mixture (32 mg./g.). A theoretical recovery of N can then be obtained in 4–5 hr.; in 2 hr. [cf. Weissman & Schoenheimer, 1941] we obtained 98·5%. We confirm the finding of Vickery (private communication) that histidine requires 8 hr. digestion for a theoretical recovery of N. In our experiments with casein and egg albumin (see Table 2) we obtained 15·3% N for each protein when the digest was heated for no longer than ½ hr. after clearing. Similar results were obtained when using the digestion mixture of Campbell & Hanna [1937].

Lysine (especially) and histidine are no doubt responsible for the much longer heating period required to obtain a maximum recovery of N.

Estimation of ammonia. The usual Pregl micro-distillation apparatus was used. Haack micro-burettes, graduated to 0·05 ml. and reading accurately to 0·01 ml., were used for the *N*/70 NaOH and HCl respectively. The latter was made up in 20% ethanol with Tashiro's indicator according to the directions of Conway & Byrne [1933].

DISCUSSION

A summary showing the reproducibility of our results is given in Table 2. It will be seen that our values for egg albumin are in agreement with those of Vickery & Shore, and slightly exceed those of Osborne and of Sörensen: we find also, in confirmation of Sörensen, that there is no significant difference in the values for the native and the heat-coagulated protein. We would tentatively suggest that the lower and irregular values recorded by Hendrix & Dennis [1938] are due in large part to the fact that they worked with anhydrous material. In our attempts to do this we have invariably found that both the native and heat-coagulated proteins are so hygroscopic that 2–3% of moisture are taken up if the sample is exposed for only a few minutes in a desiccator that has been recently opened to the air of the laboratory; in other words, the anhydrous products have desiccating properties and compete with the desiccant for any water vapour present. Anhydrous edestin does not exhibit this peculiarity to such a marked degree and we note that Hendrix & Dennis's value for this protein (18·70% N) is in excellent agreement with Osborne's and our own. Anhydrous insulin [cf. Miller & du Vigneaud, 1937] and anhydrous pepsin, on the contrary, are extremely hygroscopic and we have found it convenient to use the air-dried material. Although only small amounts of crystalline pepsin were available we have had no difficulty in obtaining comparable results by the Kjeldahl and Dumas methods (cf. Northrop's results given in Table 1). When sending the 2 samples of this protein Dr Philpot informed us that they might contain, as is usual, some non-protein-N; we mention this because a somewhat higher value for total N (15·4%) has been recorded by Calvery, Herriott & Northrop [1936]. Crystalline enzymes and protein hormones are usually isolated in small amount after an extensive procedure, so that the investigator naturally begrudges the material that must be allocated to elementary analysis. We think it timely therefore to issue a warning that such products, when anhydrous, are likely to be extremely hygroscopic and need special precautions in handling. During our investigations of egg albumin we sent samples of the heat-coagulated product to a firm of micro-analysts for determination of the moisture content by drying at 100° *in vacuo*. The results, obtained by what we believe to be a standard procedure, ranged from 6 to 7%, whereas we had ourselves obtained 11% when the product was dried at atmospheric pressure for 4 hr. at 102° in a weighing bottle, the stopper of which was replaced before removal from the oven.

In general, it will be seen that our results confirm the higher values recorded by earlier authorities who used macro-methods. Our value for amandin (18·76%) is in keeping with Osborne's surmise that his own, determined by the Dumas method, was a little too high. In the case of excelsin we had only

Table 2. *Nitrogen content of various proteins*

(Values given in parentheses indicate the ranges of analysis; D indicates Dumas analysis.)

Protein preparation	Air-dried material: Moisture content %	Air-dried material: Ash content %	No. of determinations	N, moisture- and ash-free, %
Egg albumin, native protein A	13·3	26·5	8	15·76 (15·69–15·80)
Egg albumin, heat coagulated A	11·0	0·44	7	15·76 (15·71–15·79)
			6	D 15·72 (15·69–15·84)
Egg albumin, heat coagulated B	9·8	0·5	7	15·74 (15·67–15·81)
Edestin, Sample A	11·2	0·05	7	18·65 (18·62–18·70)
Edestin, Sample B	11·1	0·2	6	18·70 (18·66–18·76)
β-Lactoglobulin	6·7	0·28	6	15·58 (15·53–15·67)
Casein	10·2	0·75	8	15·73 (15·72–15·75)
Excelsin	8·4	0·1	4	17·94 (17·84–18·02)
Amandin, Sample A	9·3	0·1	2	18·77 (18·76, 18·78)
Amandin, Sample B	8·1	0·94	2	18·75 (18·71, 18·78)
Carboxyhaemoglobin (horse), Sample A	12·7	—	2	16·80* (16·82, 16·78)
Insulin	10·0	1·24	2	15·54 (15·53, 15·54)
Pepsin, Sample A	10·87	1·2	1	14·95
			1	D 14·7
Pepsin, Sample B	9·94	1·2	1	14·78
			1	D 14·83

* Ash not deducted.

a small amount available and it was necessary to analyse the anhydrous material. The values obtained show wider variations than we expect when using air-dried material and we see no reason to doubt the slightly higher value (18·17 %) reported by Osborne & Harris [1905] for a sample of good crystalline material. Taken as a whole our results demonstrate that in recent years many investigators, whose interests have naturally been focused on the main aspects of their research, have tended to regard the determination of protein-N as a simple matter demanding no special precautions and have paid insufficient attention to the technique of Kjeldahl analysis. These findings are disturbing, for it is becoming more and more customary in amino-acid analysis, especially when only small amounts of protein are available, for the investigator to employ what he regards as moisture-free material, or alternatively, to take an unknown amount of the air-dried product and compute the weight of moisture-free and ash-free protein from a determination of total N in the hydrolysate, using as a conversion factor Osborne's or some other value for the N content of the protein. The latter procedure is clearly at the mercy of the Kjeldahl analysis, and if the technique employed leads to a low result the computed weight of protein is in error, as must also be the results of all subsequent amino-acid estimations. A similar criticism will apply to physico-chemical studies of proteins in solution, wherein the amount of protein present is computed from a Kjeldahl analysis of an aliquot.

SUMMARY

1. Attention is drawn to the fact that in recent years many protein chemists have been employing a technique for the Kjeldahl estimation of N in proteins and protein hydrolysates which gives low results; this is especially true if micro-methods have been used. Instances are cited in which recorded values for the N content of proteins are significantly lower than those obtained by earlier authorities such as Osborne & Sörensen.

2. It is shown that these low values are due to two causes: (1) The digestion period has been too short: with proteins and protein hydrolysates it is necessary to continue the heating for 8 hr. or more after the digest has cleared. (2) Certain proteins (e.g. egg albumin, pepsin and insulin), when anhydrous, are so hygroscopic that they cannot be handled without special precautions; moisture and N contents therefore should be determined, whenever possible, on separate samples of air-dried material.

3. Using the technique described the following values have been obtained for the N content of the moisture- and ash-free protein: egg albumin (native and coagulated), 15·76 %; edestin, 18·7 %; β-lactoglobulin, 15·58 %; casein, 15·73 %; amandin, 18·75 %; insulin 15·54 %; horse carboxyhaemoglobin (moisture- but not ash-free), 16·8 %.

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Critique of the Foreman Method for the Estimation of the Dicarboxylic Acids in Protein Hydrolysates

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The lime-ethanol method of Foreman [1914*a*] for the estimation of aspartic and glutamic acids in the products of protein hydrolysis has found wide application, and in the hands of later workers has undergone various modifications in detail. Foreman recited the conditions that he considered necessary for the quantitative precipitation of the dicarboxylic acids as their Ca salts from the amino-acid mixture obtained by hydrolysing the protein with HCl. He pointed out that cystine and tyrosine, as well as small amounts of chloride and basic material might also be carried down. The lime-ethanol precipitate was separated by filtration, dissolved in water, Ca^{++} removed as oxalate, Cl^- as AgCl and basic substances with phosphotungstic acid. The excess of the latter and any SO_4^{--} was next removed with baryta, the clarified solution taken to dryness and then extracted with glacial acetic acid. The two dicarboxylic acids remained insoluble and were estimated by differential analysis for C, H and N. The acetic acid extract contained any pyrrolidonecarboxylic acid formed from glutamic acid during the various treatments applied, as well as other products, and the former was estimated by the increase in amino-N following strong HCl hydrolysis. In an addendum to his paper he stated that in the light of subsequent work [Foreman, 1914*b*] he believed that if the temperature for all processes of concentration was kept below 45° the amount of pyrrolidone formation would be reduced to a negligible amount.

Dakin [1918; 1920; 1923] hydrolysed the protein with H_2SO_4, removed the mineral acid with baryta, extracted the monoamino-acids with butanol and then precipitated the dicarboxylic acids under the Foreman conditions as their Ba salts, glutamic acid subsequently being isolated as the hydrochloride, and aspartic acid via the Pb salt. Jones & Moeller [1928] applied the Foreman method direct; in most cases they used the Ba salts and found it necessary to make a preliminary precipitation with phosphotungstic acid to prevent the Ba dicarboxylates being heavily contaminated with basic material. These latter workers, who isolated the glutamic acid as the hydrochloride and aspartic acid as the Cu salt, found that in most cases it was necessary to repeat the original precipitation of the Ba salts to free the mixed products from monoamino-acids such as glycine and tyrosine. Calvery [1931] followed the general procedure of Jones & Moeller and pointed out that losses might be incurred if the monoamino-acids were removed by a preliminary extraction with butanol, as recommended by Dakin. The results of all these workers showed a marked improvement over those obtained by the older ester method of Fischer.

In this laboratory some preliminary dicarboxylic acid analyses of cocksfoot protein were made by Miller [1936]. The Dakin procedure for reasons not then apparent gave low results, while that of Jones & Moeller was by no means satisfying, for much of the N in the Ba precipitate remained unidentified. More systematic investigations of the products obtained from various proteins by HCl hydrolysis were started the following year to ascertain the respective merits of the Ca and Ba salts, the best conditions for the first and repeat precipitations with ethanol, and details of technique for the isolation of glutamic acid as its hydrochloride and of aspartic acid as its Cu salt. These were followed by an analysis of myosin [Sharp, 1939], which demonstrated the superior selectivity of the Ca over the Ba salts when precipitated in the presence of much basic-N* and confirmed Calvery's finding that a preliminary extraction with butanol to remove monoamino-acids may lead to loss of dicarboxylic acids, indicating that the precise conditions under which this extraction can be successfully applied are not readily defined. Using the experience thus gained, reasonably satisfactory dicarboxylic acid analyses were made of a series of protein preparations from forage crops [Chibnall, 1939]. On the completion of this latter work we were free to devote our attention to the more interesting problem [Chibnall, 1942; Chibnall, Rees & Williams, 1943] of perfecting our methods of analysis so as to obtain results for both aspartic acid and glutamic acid which we could be reasonably certain were accurate to about 2%.

* In a private communication to one of us (A. C. C.) Mr Foreman stated that in 1914 he was aware of the superior selectivity of the Ca salts and that his choice was deliberate. The contrary opinion of Town [1941] was based on experiments with gliadin, which contains very little basic-N.

EXPERIMENTAL

We analysed a number of proteins of known purity by the procedure then in current use in this laboratory. The protein (40–50 g.) was hydrolysed with 20% HCl and then passed twice in succession through the lime-ethanol treatment, using each time an aqueous volume of 400 ml. and 9 vol. of 95% ethanol. Glutamic acid hydrochloride and Cu aspartate were then separated alternately until no more crystalline material could be obtained from the mother liquor. The lime-ethanol treatment, followed by separation of the crystalline derivatives, was then applied another 2 or 3 times, generally from aqueous volumes of 100, 25 and 20 ml. respectively. Later we made an analysis of various normal and malignant tissue proteins, and in the published account of that work the above-mentioned procedure was described in some detail [Chibnall, Rees, Williams & Boyland, 1940].

2. The Cu salt of natural *d*(+)-aspartic acid crystallizes from water in clusters of fine blue needles; these can be washed with cold water and dried via ethanol and ether, when they attain a constant air-dry weight in a few hours. The moisture content, determined by heating to 130° for 3 hr., is 29·4%, corresponding to 4·5 H_2O, and if the acid hydrochloride and Cu aspartate are being removed alternately, the salting out of the former is hindered by the $CaCl_2$ present and the product is often contaminated with a small amount of $CaSO_4$. These difficulties, which can be overcome by treatment, would not arise when working to the original conditions of Foreman, as he removes both Cl^- and SO^{++} at an early stage; nevertheless according to our experience the removal of these ions should be delayed until the maximum amount of Cu aspartate has been separated, otherwise heavy losses of aspartic acid may be incurred on account of the insolubility of Ag and Ba aspartates.

Table 1. *Aspartic and glutamic acid content of some proteins*

	Total N ash- and moisture-free %	New analysis		Literature		
		Glutamic acid %	Aspartic acid %	Glutamic acid %	Aspartic acid %	Authority
Casein (1)	15·72	20·7	6·14	21·6	4·1	Dakin [1918]
Casein (2)	15·72	20·7	6·10	—	—	
Edestin (1)	18·65	18·6	10·55	19·2	10·2	Jones & Moeller [1928]
Edestin (2)	18·65	19·36	10·95	—	—	
Cattle haemoglobin	16·65	5·83	8·03	3·5	6·4	Bergmann & Niemann [1937]
Egg albumin (2)	15·75	14·0	7·02	14·0	6·1	Calvery [1931]

The results given in Table 1 show that the method we were then using for the estimation of aspartic acid was superior to those hitherto employed, but that we had affected no improvement in the estimation of glutamic acid. Our cumulative experience nevertheless convinced us that both these estimations were still far from being quantitative, as will be apparent from the following observations:

1. When the precipitated Ca salts are redissolved in water Ca^{++} cannot be removed quantitatively unless an extended treatment be applied. This is due to the fact that at the stage prior to the addition of lime (which generally contains traces of sulphate) the hydrolysate consists of a mixture of amino-acid hydrochlorides, and on precipitating the Ca salts Cl^-, roughly equivalent to about 20–30% of the HCl present according to our experience, is carried down presumably in the form of the additive compounds noted by Anslow & King [1927] and by Cannan & Kibrick [1938]. Consequently on subsequent removal of Ca^{++} as oxalate a stage is ultimately reached when the regenerated HCl prevents further precipitation of Ca oxalate and $CaCl_2$ remains in solution. At a later stage, when glutamic acid hydrochloride is being removed alternately with the Cu aspartate, the salting out of the former is hindered by the $CaCl_2$ present and the product is often contaminated with a small amount of $CaSO_4$. These difficulties, which can be overcome by treatment, would not arise when working to the original conditions of Foreman, as he removes both Cl^- and SO^{++} at an early stage; nevertheless according to our experience the removal of these ions should be delayed until the maximum amount of Cu aspartate has been separated, otherwise heavy losses of aspartic acid may be incurred on account of the insolubility of Ag and Ba aspartates.

2. The Cu salt of natural *d*(+)-aspartic acid crystallizes from water in clusters of fine blue needles; these can be washed with cold water and dried via ethanol and ether, when they attain a constant air-dry weight in a few hours. The moisture content, determined by heating to 130° for 3 hr., is 29·4%, corresponding to 4·5 H_2O, and if the atmospheric humidity is low this may fall to about 28·5% but not lower. Adequate data in support of this statement, which confirms the early work of Ritthausen [1869] and of Hofmeister [1877], will be given in a subsequent communication, and it may be pointed out here that heating the air-dried salt at 140–150° to constant weight, a procedure employed by Abderhalden & Weil [1911] to demonstrate that it contained 5 H_2O, leads to decomposition. We are unable to substantiate the claim of Bergmann & Niemann [1937] that when the air-dried salt is heated for 6 hr. at 105° the moisture content is reduced to 15·62%, equivalent to $2H_2O$. (Found: moisture 14·4, 14·9%.) The Cu salt of racemic aspartic acid crystallizes in fine needles of the same habit and colour as the natural salt and has about double the solubility of the latter in water (30 mg./100 ml.) at 20°: air-dried it also contains moisture equivalent to 4·5 H_2O. When pure the blue colour of both the air-dried salts has a characteristic violet tinge that persists even when the material is finely ground. In the many dicarboxylic acid analyses that have been discussed above it was seldom that products of this purity were obtained without resort to recrystallization

and in this respect our experience appeared to be similar to that of Jones & Moeller [1928], who mention that the moisture content of their air-dried products often varied from the equivalent of 4·5 H_2O. Under the microscope our products showed the presence of irregular nodular masses as well as the typical needles of Cu aspartate, and the greater the proportion of the former the less did the colour of the finely ground material match that of pure Cu aspartate. Table 2 gives the analysis of two such products, Cu and N being determined on the anhydrous material.

Table 2. *Characteristics of some Cu-aspartate fractions*

Sample	Colour	H_2O %	Cu %	N %
(1) From edestin	Dull blue	27·8	32·6	7·35
(2) From edestin	Dull blue with distinct greenish tinge	22·0	29·7	7·59
(3) Pure air-dried Cu aspartate	Blue with violet tinge	29·4	32·7	7·19

Sample 1 might well be regarded as pure, yet on recrystallization under appropriate conditions some impurity was removed. In the analyses quoted in Table 1 it has been necessary to recrystallize all the samples of Cu aspartate except the first two main crops from each hydrolysate of casein. The rejected material contained Cu aspartate however and the quoted values for the aspartic acid content of proteins concerned must have been low. To appreciate the steps which led to the recognition of this impurity, and its avoidance in later work, it is necessary to recite briefly the method in use at the time for the separation and purification of the Cu aspartate.

When the solution of mixed amino-acids recovered from the lime-ethanol precipitate, or of the mixed hydrochlorides obtained from them after a preliminary removal of glutamic acid hydrochloride, was treated at the boil with excess $CuCO_3$, a fine scum of varying shades of olive and grey green was generally the first product to separate. If this occurred before the removal by filtration of the excess $CuCO_3$ the Cu aspartate that crystallized out from the filtrate on cooling was fairly pure, as in sample 1 (Table 2). More often, however, the scum did not separate until after filtration, and very occasionally it was possible to remove this by another filtration before crystallization of the Cu aspartate. The scum thus removed was always green and in some cases, especially when small end fractions were being dealt with, it often contained traces of yellowish material. Usually, however, the scum and aspartate separated together and in the case of end fractions very impure products were thereby obtained, as in sample 2 (Table 2). This particular sample was dissolved in hot water with the aid of a few drops of HCl, boiled with excess $CuCO_3$ and filtered hot. The filtrate, as it cooled, deposited a green flocculent precipitate which was removed and washed in the usual way. The yield was 18·3 % and, dried at 100°, the product contained 29·9 % Cu and 7·6 % organic-S. This latter finding gave us at once the clue to our difficulty, for it suggested that during the lime-ethanol treatment cystine was undergoing dismutation [Lavine, 1936] to give the sulphinic or sulphonic acid, perhaps also cysteic acid, all of which dibasic acids give insoluble Ca salts under these conditions. Our experience with casein, which was a very pure sample containing only 0·24 % cystine, tended to confirm this, for the first two large crops of Cu aspartate did not need recrystallization. It will be recalled that Foreman [1914*a*] showed that cystine would be precipitated under the conditions of his lime-ethanol procedure. To obtain further evidence we oxidized some cystine by the method of Friedmann [1903], using insufficient Br to effect a quantitative conversion to cysteic acid. On making the Cu salt according to his directions a scum separated at once from the hot filtrate, and on elutriating this with water we obtained a series of products ranging in colour from dull blue to olive and grey green, typical of those we had encountered in our dicarboxylic acid analyses.

3. Foreman mentioned that tyrosine would be precipitated with the Ca salts of the dicarboxylic acids and we accordingly made it a custom to remove as much of this amino-acid as possible by direct crystallization at the stage immediately prior to the lime-ethanol treatment. Even so the material recovered from the Ca precipitate, after being worked over to remove the major part of the glutamic and aspartic acids, would always yield small amounts of crystalline tyrosine on appropriate treatment [cf. Chibnall *et al.*, 1940]. Moreover, the end fraction, from which no further amount of crystalline amino-acids or derivates could be separated, always gave a strong Millon test for tyrosine and also a strong Sakaguchi test for arginine. We had also noted that when these dicarboxylic acid fractions, after being reduced *in vacuo* to a small volume, were concentrated on the water-bath in the presence of HCl to the incipient crystallization of glutamic acid hydrochloride some substance was decomposed to give small amounts of NH_3. It is possible, from the work of Damodaran & Ramachandran [1941], that the substance concerned was serine, although our experience suggests that the free amino-acid is much more stable to hot strong acid than its phosphoric acid ester. Our final end fraction therefore, which reduced to an intractable syrup, must have contained tyrosine, arginine, serine and perhaps also its decomposition products,

as well as cystine and its dismutation products. It is from these end fractions, not always as well attenuated as those obtained during the analyses referred to in Table 1, that certain earlier workers have isolated what they have believed to be hydroxyglutamic acid. As we show in Appendix 2, we encountered such products in a few of our earlier analyses, but these always yielded relatively large amounts of aspartic acid and sometimes of glutamic acid on appropriate treatment.

Now as experience was gained during the course of this research the relative amount of N that remained in the dicarboxylic acid end fraction became progressively less, with an accompanying increase in the amount of aspartic acid isolated. The improvement was due to the more effective removal of the various amino-acids mentioned above, whose presence can retard the crystallization of Cu aspartate. This salt is very insoluble in cold water, yet 0·33 g. will dissolve readily when stirred into 100 ml. of water at 20° containing 1·9 g. of Cu serine. Such solubility effects illustrate the underlying fallacy of the so-called Brazier [1930] method for the separation of the products of protein hydrolysis. This method, in fact, was first suggested by Town [1928; 1936; 1941] who has recently drawn attention to its limitations.

All the above-mentioned observations convinced us that our final end fraction, though representing no more than about 1 % of the total protein-N, still contained aspartic acid and perhaps also glutamic acid, and the first problem ahead of us was the complete removal of cystine at an early stage, when the hydrolysate was still acidic, so as to prevent its dismutation during the lime-ethanol treatment.

Removal of cystine as cuprous mercaptide prior to the lime-ethanol precipitation

During the course of our research on the glutamic acid content of normal and malignant tissue proteins we had occasion to repeat some of the experiments of Kögl & Erxleben [1939]. These workers hydrolysed the tissue material with HCl and after removing the excess of acid in the usual way they clarified their hydrolysate by treatment with Cu_2O, a reagent first introduced by Hlasiwetz & Habermann [1873], who found it very effective for the purpose and also a cheap substitute for Ag_2O in the removal of the residual chloride. Certain of our hydrolysates clarified in this way were afterwards passed through the lime-ethanol procedure, and we observed that the various crops of Cu aspartate subsequently isolated were free from all traces of the scum mentioned earlier, were of high purity and did not need recrystallization. This suggested to us the possibility, which we found subsequently had already been demonstrated by Rossouw & Wilken-Jorden [1935], that the Cu_2O, or more correctly the Cu_2Cl_2, was reducing the cystine to cysteine, which was then being precipitated as the cuprous mercaptide [Vickery & White, 1932]. At this time we already had in view an outline of the extended procedure that we hoped to apply to a dicarboxylic acid and base analysis of proteins, and Rossouw & Wilken-Jorden's treatment for the removal of cystine was unsuitable for our purpose in that it would introduce reagents whose subsequent removal would present difficulties. The direct use of Cu_2O was accordingly investigated in some detail, the work being completed and the results applied to edestin, egg albumin and zein before the publication of a paper on somewhat similar lines by Lucas & Beveridge [1940].

A short account of the experiments leading to the procedure finally adopted is given in Appendix 1. To effect the complete precipitation of the cystine as the cuprous mercaptide of cysteine it is necessary to add an excess of Cu_2O, thereby bringing about an almost complete removal of HCl as Cu_2Cl_2 and reducing the acidity of the hydrolysis mixture to about *p*H 3·5. As a result a small amount of the more insoluble monoamino-acids, viz. methionine, tyrosine and probably also leucine and phenylalanine, separate as such, or as copper complexes, and contaminate the mercaptide precipitate. Careful search, however, has shown that there is no loss of dicarboxylic acids or arginine. The new treatment has been applied to analyses of edestin, egg albumin and β-lactoglobulin, full details of which are given in a later paper [Chibnall *et al.*, 1943]. As was to be expected, there was a great improvement in the purity of the Cu aspartate subsequently isolated, and only very occasionally has it been necessary to recrystallize the product before satisfactory analytical data could be obtained. In these analyses a more complete removal of the interfering monoamino-acids such as tyrosine and serine carried down in the initial precipitate of the Ca dicarboxylates was effected by applying the third and subsequent lime-ethanol treatments from a larger aqueous volume than had been our custom in the past.

The results for edestin and egg albumin are given in Table 3 under the heading '1st complete lime-ethanol treatment'. The new procedure has given in both cases a satisfactory increase in aspartic acid, but no increase at all for glutamic acid, suggesting that the separation of this amino-acid as hydrochloride was well nigh complete under the old conditions. That both the dicarboxylic acids have been effectively removed is shown by the fact that in the egg albumin analysis the ultimate mother liquor contained only 0·95 % of the total protein-N and of this some 40 % was non-amino-N, almost certainly basic-N since precautions had been taken to prevent the conversion of glutamic acid to pyrrolidone-

carboxylic acid. Moreover, the Millon test for tyrosine and the Sakaguchi test for arginine were strongly positive. These results convinced us that we had brought our treatment for the isolation of glutamic and aspartic acids from the lime-ethanol precipitate to workable limits; even so we could not regard our new values as quantitative until we had ascertained whether any significant amount of either of the respective Ca salts had escaped precipitation and had thus passed into the lime-ethanol filtrate.

Examination of the lime-ethanol filtrate for dicarboxylic acids

As will be more fully discussed in Appendix 3, the Ca salts of both aspartic acid and glutamic acid, under the conditions of our lime-ethanol procedure, have residual solubilities that are subject to the concentration and the degree of racemization of the acid concerned and to the concentration of Cl^-. At an early stage in the research, before the interdependence of these three factors had been established, we had determined solubility factors by submitting an aqueous solution of each natural acid to the necessary lime-ethanol treatment and these suggested that our values for aspartic acid and glutamic acid should be raised by about 0·3 and 0·7 % of the weight of protein respectively. Such solubility effects were larger than we had anticipated and we were not willing to concede them without a more direct proof based on the isolation of the two amino-acids from the lime-ethanol filtrates.

Accordingly we undertook an extended analysis of gliadin. After hydrolysis with 20 % HCl the hydrolysate was reduced in volume and saturated with gaseous HCl; this caused the separation of a large crop of glutamic acid hydrochloride which was subsequently recrystallized from water [Osborne & Guest, 1911]. The collected mother liquors were then passed through the lime-ethanol procedure in use at the time; as this did not include the preliminary application of Cu_2O the various small crops of Cu aspartate were heavily contaminated with the scum mentioned in an earlier section, and needed careful recrystallization. When the lime-ethanol precipitate had been worked over to completion we had isolated in all 45·82 % of glutamic acid and 1·34 % of aspartic acid (*vide* Table 3), values that from the standpoint of analytical technique are comparable with those of edestin (2) quoted in Table 1. Now at the stage when the first precipitation of the Ca salts was made the hydrolysate contained amino-acids, other than dicarboxylic acids, representing 48 % of the total protein-N. All of these other amino- (and imino-) acids, as well as any residual dicarboxylic acids due to solubility effects, passed into the collected lime-ethanol filtrates, and our object was to devise methods whereby these other amino-acids could be progressively removed so as to give a final mother liquor in which the residual dicarboxylic acids would be present in sufficient concentration to permit their isolation by a repeat application of the lime-ethanol procedure. The various filtrates and the final dicarboxylic acid enriched fraction were collected, reduced *in vacuo* to remove ethanol and then freed from inorganic ions (Ca^{++}, Cl^- and traces of $SO_4^=$). The clarified solution was next concentrated to incipient crystallization whereby several successive crops of mixed mono-amino-acids (leucine-tyrosine-phenylalanine) were removed. The mother liquor was then extracted 12 times successively with an equal volume of warm butanol [Miller, 1936] to remove further amounts of monoamino-acids, a treatment that extracts but little proline. This was followed by a precipitation under the usual conditions with phosphotungstic acid, which carried down the bases and much proline. The filtrate was freed from phosphotungstic acid by extraction with amyl alcohol-ether and from excess HCl by repeated evaporation *in vacuo*. At this stage the solution contained only 12·8 % of the total protein-N in the form of amino-acids other than dicarboxylic acids, and on repetition of the lime-ethanol procedure we were able to isolate a further 1·1 % of glutamic acid and 0·06 % of aspartic acid. This experiment demonstrated quite clearly the relatively large solubility of Ca glutamate under the conditions of our modified Foreman lime-ethanol procedure, especially when some of the acid is present in racemized form, as in this gliadin hydrolysate [Chibnall *et al.*, 1940, Table 1]. The solubility of Cu aspartate on the contrary appeared to be small, a result accepted at the time with reserve on account of the omission of the Cu_2O procedure and of possible losses arising from the conditions under which the base precipitation with phosphotungstic acid had been performed.

Having made this preliminary survey we were in a position to devise a more suitable, albeit a more laborious, procedure which has been applied in turn, with minor modifications suggested by experience, to hydrolysates of zein, edestin, egg albumin and β-lactoglobulin. Full details of the last three of these analyses are given in a later paper [Chibnall *et al.*, 1943] and all that need be quoted here are the summarized results for edestin and egg albumin given in Table 3, which permit a direct comparison with the earlier ones given in Table 1. In the new analyses it was possible to reduce the amount of non-dicarboxylic acid-N from about 70 % to about 14 % of the total protein-N before repetition of the final lime-ethanol procedure and, as in the case of gliadin, substantial amounts of (partially racemized) glutamic acid were subsequently isolated as hydro-

chloride. The new procedure has also led to the isolation, as Cu aspartate, of significant amounts of aspartic acid. The analyses of zein and β-lactoglobulin gave similar results and, as will be discussed in later papers, there is reason to believe that our present procedure gives values for the aspartic acid and glutamic acid contents of the proteins concerned that are accurate to within 2%. These findings show without any equivocation that the lime-ethanol method of Foreman, as applied by us and by all other workers in the past, must have given results that were far from quantitative and hence of no value whatsoever from the point of view of the Bergmann-Niemann hypothesis.

Table 3. *Redetermined aspartic and glutamic acid contents of some proteins*

Protein	Method of isolation	Non-dicarboxylic acid-N present when Ca salts were first precipitated (% total protein-N)	Glutamic acid (% of protein)	Aspartic acid (% of protein)
Gliadin	Direct as hydrochloride	—	33·53	—
	1st complete lime-ethanol treatment	48·0	12·29	1·34
	2nd complete lime-ethanol treatment	12·8	1·10	0·06
		Total	46·92	1·40
Edestin (3)	1st complete lime-ethanol treatment	68·8	19·34	11·24
	2nd complete lime-ethanol treatment	13·9	1·19	0·71
		Total	20·53	11·95
Egg albumin (2)	1st complete lime-ethanol treatment	70·39	13·89	7·74
	2nd complete lime-ethanol treatment	21·3	2·11	0·36
	3rd complete lime-ethanol treatment	12·0	0·0	0·03
		Total	16·00	8·13

Appendix 1. Procedure for Removal of Cystine as the Cuprous Mercaptide of Cysteine. (By K. Bailey)

For these experiments wool and edestin were chosen because they contained very high and average contents of cystine respectively. The wool was taken from a raw Cotswold fleece, cleaned and prepared in a manner previously described [Bailey, 1937]: it contained 0·13% ash and, ash- and moisture-free, 16·55% N. Both this and the edestin were hydrolysed for 24 hr. with 20% HCl, and the excess HCl removed by evaporation under reduced pressure in the usual way. The hydrolysate was then made up to standard volume and aliquots withdrawn for determination of N by micro-Kjeldahl and of S by alkali fusion [Bailey, 1937]. Cu_2O was prepared by reduction of Fehling's solution with glucose, washed free from all inorganic ions and dried via ethanol and ether.

For the mercaptide precipitation solutions containing 0·7–0·9 g. N/100 ml. in the case of wool, and 0·8–1·1 g. N/100 ml. in the case of edestin, were employed. Portions of the hydrolysate were treated with Cu_2O under varying conditions, which are described below in apposition to individual experiments; after cooling to 10° the precipitate was centrifuged and washed 6 times with warm water. The washings were collected, reduced *in vacuo*, cooled to 10°; the small amount of material that separated was centrifuged off and added to the main precipitate. For N and S analyses the latter was either dissolved in hot HCl or dried in acetone and ground to a fine powder, depending on the amount available.

Wool. The following experiments were performed:

(1*a*) The hydrolysate was shaken with excess Cu_2O at 15° for 30 min., cooled to 10° and the mercaptide precipitate separated without delay.

(1*b*) As in (1*a*), but the reaction was allowed to proceed overnight before the separation.

(1*c*) As in (1*a*), but the reaction temperature was raised to 45° and the excess Cu_2O added in small portions over a period of 30 min.

(2) The Cu_2O was added slowly to the hydrolysate at 45°, small samples of which were removed from time to time and clarified by centrifuging to test whether the addition of a further amount would produce any visible separation of mercaptide. In this way the considerable excess of Cu_2O added in 1*a*–1*c* was avoided and the supernatant liquid was green instead of deep blue.

(3) This was a large-scale repetition of (1*c*) under conditions considered most suitable for the purpose, and was designed as the starting-point of an extended amino-acid analysis. The hydrolysate, containing 18·4 g. N in 2 l., was maintained at 45–50° whilst a suspension of Cu_2O in 500 ml. of water was poured in slowly with rapid stirring until a test sample showed that excess Cu_2O was present and the supernatant liquid was deep blue. Stirring was continued for another hour and the mixture was then kept at 10° overnight.

The results are given in Table 4. It will be seen that for the complete removal of cystine the reaction temperature must be raised to 45°, and an excess of Cu_2O must be present (Exp. 1*c*, 3). Under such conditions the acidity of the solution is reduced to about *p*H 3·0–3·5 by removal of HCl, and the supernatant liquid becomes deep blue. The N carried down is in excess of that due to cystine, a point discussed later.

Edestin. Based on the experience gained with wool hydrolysates the following experiments were performed:

(4) The conditions were as in (3).

(5) The starting-point of a large-scale comprehensive dicarboxylic acid and base analysis described in detail in a later communication [Chibnall *et al.*, 1943]. The conditions were as in (3).

(6) The hydrolysate was heated with a small amount of Zn and HCl for 1 hr. and then neutralized with NaOH at *p*H 6. The humin thus precipitated was separated and the filtrate adjusted to *p*H 4 with HCl. Excess Cu_2O was then added at 45° and the mixture agitated for 1 hr.

(7) The procedure of Graff, Maculla & Graff [1937] was employed. The hydrolysate was first reduced with Zn and HCl, adjusted to *p*H 5 with Na acetate, humin removed, the solution adjusted to *p*H 4 with acetic acid and then treated with Cu_2O. The mercaptide precipitate was washed with a citrate-acetate buffer at *p*H 6.

The results given in Table 4 show that in Exp. 4 and 5 the mercaptide-S is greatly in excess of the non-methionine-S, assumed to be cystine-S, which amounts to 43·5% of the total S. Under the conditions of (6) and especially of (7) the mercaptide-S approaches the expected value for cystine-S, though in the latter case the mercaptide-N is higher than the equivalence claimed by Graff *et al.* [1937].

The ideal procedure for our large-scale amino-acid analysis would have been one in which the precipitate contained only the mercaptide of cysteine, Cu_2Cl_2 and excess Cu_2O, together with any humin not already removed at an earlier stage. That of Graff *et al.* comes nearest to fulfilling these conditions, but was unsuitable for our purpose in that it would introduce into the hydrolysate reagents whose subsequent removal would present difficulties. The technique used in Exp. 3, 4 and 5 was satisfactory in so far as removal of cystine was concerned, yet at the time these were performed we were puzzled by the large excess of S and N carried down. In this connexion our findings were in full keeping with those published recently by Lucas & Beveridge [1940], who used a somewhat different technique and found that their mercaptide precipitate was heavily contaminated with extraneous N. As these workers determined cystine-S and not total organic-S they were apparently unaware that non-cystine-S was carried down.

The excess organic-S in our mercaptide precipitates was clearly due to methionine, yet the reason for this was not immediately apparent. When a solution of this amino-acid was treated with Cu_2O under comparable conditions only 4% of the original N was carried down. Moreover, Bailey [1937] had already shown that the methionine content of a protein first hydrolysed with HCl is the same as that of the native protein when the determination is made by the volatile iodide method; secondary reactions, such as homocystine formation during hydrolysis, were thus excluded. It was not until the mercaptide precipitates obtained in our large-scale analyses of egg albumin and edestin had been submitted to detailed investigation that we were able to explain why they contained more organic-S and N than had been expected. The reason is that the addition of excess Cu_2O to the hydrolysate, which is necessary for the complete precipitation of the cystine as the cuprous mercaptide of cysteine, removes the major part of the HCl present as insoluble Cu_2Cl_2 and the acidity of the solution is thereby reduced to about *p*H 3. Such conditions are favourable for the direct crystallization of certain of the more insoluble monoamino-acids, especially methionine, tyrosine, leucine and perhaps phenylalanine [Hill & Robson, 1934; Baptist & Robson, 1940], and it is these monoamino-acids, or possibly their copper complexes, together with humin, that are responsible for the excess N and organic-S present in the mercaptide precipitates. Appropriate treatment has shown that arginine [Lucas & Beveridge, 1940] and the dicarboxylic acids are not concerned, a matter of importance in our work.

Table 4. *Composition of various mercaptide precipitates*

Exp. no.	Total S minus free SO_4-S g.	Mercaptide-S as % total protein-S	Filtrate from mercaptide: S minus SO_4-S as % total protein-S	Total N in hydrolysate g.	Mercaptide-N as % total protein-N	Cystine-N as % total protein-N
(*a*) Wool						
1*a*	0·0716	89·8 (diff.)	10·2	—	—	—
1*b*	0·0716	89·6	13·7	0·3748	10·03	8·32
1*c*	0·0716	100·6	0·6	0·3748	11·74	8·32
2	0·640	90·6	9·4 (diff.)	3·28	11·6	8·32
3	3·607	97·3	3·6	18·41	12·4	8·32
(*b*) Edestin						
4	0·039	74·1	29·0	0·7903	2·62	0·85
5	0·541	67·3	32·7 (diff.)	11·554	2·20	0·85
6	0·027	51·9	48·1 (diff.)	0·5745	1·83	0·85
7	0·027	45·5	54·5 (diff.)	0·5745	1·34	0·85

Appendix 2. On the Possible Presence of Hydroxyglutamic Acid in Protein Hydrolysates

As Nicolet & Shinn [1942] state in a recent paper, the history of β-hydroxyglutamic acid is a peculiar one. In their introduction, these workers give a review of the position that had developed since Dakin [1918] announced the isolation of this substance from casein, and it is unnecessary to recapitulate the various evidences in the present communication, except to point out that many workers who have attempted to prepare β-hydroxyglutamic

acid for experimental purposes have been unsuccessful or have obtained products which they believed to contain considerable amounts of the desired substance, but have not adequately characterized it. Applying their new periodic acid oxidation method for the estimation of threonine and serine in protein hydrolysates, together with that of Van Slyke, Hiller, MacFadyen, Hastings & Klemperer [1940] for 'total hydroxyamino-acids', Nicolet & Shinn [1942] find no evidence for the presence of hydroxyglutamic acid in the three milk proteins, casein, β-lactoglobulin and lactalbumin. In view, however, of the claim of Gulland & Morris [1934] that this hydroxyamino-acid is indeed present in casein, albeit only to the extent of 0·3% as opposed to Dakin's original 10·5%, Nicolet & Shinn were not prepared to deny its existence in this protein, though they maintained that the amount present cannot be greater than that claimed by Gulland & Morris. Meanwhile Dakin [1941] has stated that further research on the acid that he isolated from casein, particularly his failure to convert it to ketoglutaric acid, or glutaconic acid derivatives, and its limited reaction with periodic acid, make it seem certain that his product cannot be a β-hydroxy-acid. This admission of Dakin may be regarded as closing the issue concerning β-hydroxyglutamic acid, yet the position as it now stands is not entirely satisfactory, for Dakin is exploring the possibility that his product is some other new amino-dicarboxylic acid. This raises at once a matter of great importance to those who are interested in the number of free carboxyl-groups in the intact protein molecule, for these can be computed on the one hand from electrometric titration of the protein itself and on the other hand from dicarboxylic acid and amide-N analysis after hydrolysis [cf. Chibnall, 1942]. Consequently if Dakin's product should indeed be a new dicarboxylic acid instead of, as we believe, a mixture of known amino-acids and products derived from them, it must be taken into account in drawing up an estimate of total dicarboxylic acids given on hydrolysis

When we embarked on our extended investigations of the Foreman method we naturally made careful search for hydroxyglutamic acid by the glacial acetic acid and Ag salt procedure of Dakin. In our early experiments we frequently obtained, like other workers, syrups that proved intractable. Yet occasionally, and in general by procedures different from that of Dakin, we separated crystalline or granular products whose composition suggested the possible presence of this hydroxy-amino-acid; on further treatment, however, these always yielded significant amounts of aspartic acid and often of glutamic acid. Later, when we had evidence that the Foreman lime-ethanol treatment was bringing about the dismutation of cystine to give dibasic acids, we found that all of our 'hydroxyglutamic acid' fractions contained significant amounts of organic-S and hence of cystine dismutation products, which were clearly in part responsible for the fact that the fractions often gave values for C, H and N which approximated closely to those of hydroxyglutamic acid itself. Since the introduction of the Cu_2O treatment and the development of our present procedure for the separation of aspartic and glutamic acids we have not encountered any fractions of this type whatsoever and are convinced that all the proteins that we have analysed, viz. edestin, egg albumin, β-lactoglobulin, zein, gliadin and casein, do not contain any other dicarboxylic acid. We believe that the products isolated by Dakin, and by Gulland & Morris, were mixtures of aspartic acid (especially), glutamic acid, dibasic dismutation products of cystine, serine and serine decomposition products, the proportion in which these occur varying from sample to sample. To enable us to elaborate the thesis further it will be convenient to give first of all in some detail the separation and subsequent analysis of certain 'hydroxyglutamic acid' fractions obtained in three of our earlier dicarboxylic acid analyses.

Egg albumin. This analysis was made in 1938, and the final results have already been quoted in Table 1. 51·193 g. of air-dried coagulated material, equivalent to 49·8 g. of moisture- and ash-free protein, were hydrolysed with 400 ml. of 20% HCl for 24 hr. and the excess HCl removed by repeated evaporations *in vacuo*. The acid humin was then removed, the solution and washing reduced to 500 ml., lime added to *p*H 6·8–7·0 and the alkali humin removed. The clarified solution and washing were then reduced to 200 ml. and left overnight at 0°: 1·098 g. crude tyrosine separated and were removed. The lime-ethanol treatment of Foreman was then applied twice in succession from an aqueous volume of 500 ml., the precipitated Ca salts filtered and washed, redissolved in water and Ca^{++} removed. Glutamic and aspartic acids, equivalent to 11·85 and 5·91% of the weight of protein (w/p), were separated as the hydrochloride and Cu salt respectively, and the clarified mother liquor passed through the lime-ethanol treatment from an aqueous volume of 110 ml. The precipitated material was freed from Ca^{++} as before and the clarified solution reduced to 50 ml. This was allowed to stand under slightly reduced pressure in a desiccator, when 3 crops of crystalline material (0·38, 1·09 and 0·404 g. respectively) separated and were removed. The collected material contained tyrosine (≡0·14% w/p by colorimetric estimation) and on treatment gave glutamic acid (≡1·04% w/p) and aspartic acid (≡0·86% w/p) as hydrochloride and Cu salt respectively.

The mother liquor, after removal of the above-mentioned 3 crops, was reduced to a small volume and treated with ethanol. Amorphous material (fraction 1) separated, which was washed with ethanol and ether and then dried to constant weight *in vacuo* (1·716 g.). It was non-hydroscopic, was freely soluble in cold glacial acetic acid, gave the colour test for tyrosine and contained 2·4% ash ($CaSO_4$); other analytical data are given in Table 5 and are reminiscent of those given by the 2 samples of impure S-containing nitrogenous acids isolated from casein by Gulland & Morris, one of which contained C, 31·3; H, 5·5; N, 10·6; S, 6·7% respectively. Fraction 1 contained 9·02% N, all in the amino-form, but after heating for 1 hr. at 120° the latter value had fallen to 5·72%, reminiscent of Dakin's hydroxyglutamic acid. Titration to *p*H 7·3 (blue tint with bromothymol blue) showed that only 68·4% of the N could be referred to dicarboxylic acids [*vide* Chibnall *et al.*, 1940]. 1·674 g. of fraction 1 yielded by the usual treatment 0·2571 g. of aspartic acid and 0·7114 g. of glutamic acid as Cu salt and hydrochloride respectively. The clarified mother liquor was passed through the lime-ethanol procedure, using an aqueous volume of 10 ml., the precipitate removed, freed from Ca^{++} and the solution reduced to 3 ml. On standing 0·244 g. of crystalline material (fraction 2) separated and was removed. The product was insoluble in glacial acetic acid and gave a positive colour test for tyrosine. The lime-ethanol filtrate was freed from ethanol and Ca^{++}, joined with the mother liquor from fraction 2 and taken to dryness *in vacuo*. The product (fraction 3, wt. = 0·4733 g.) was slightly hygroscopic, dissolved freely in glacial acetic acid, contained 4·5% ash ($CaSO_4$) and gave a strong colour test for tyrosine. Fraction 1 therefore,

which was freely soluble in glacial acetic acid, had yielded aspartic acid (≡0·345% w/v), glutamic acid (≡0·86% w/v), fraction 2 which was insoluble in glacial acetic acid, and fraction 3 which was freely soluble in glacial acetic acid. Fractions 2 and 3 were not investigated in further detail, but their analyses, quoted ash-free in Table 5, and especially their mode of preparation, show that they must be mixtures of several products.

Protein material from normal ox heart. The analysis of this material (47·2 g.) has already been described in detail [Chibnall *et al.*, 1940]. The amino-acid solution recovered from the Ca salts precipitated in the 5th lime-ethanol treatment was reduced to a small volume *in vacuo*; on standing 0·1232 g. of crude tyrosine separated. The mother liquor was then concentrated slowly to a syrup in a vacuum desiccator and after 2 days a few drops of ethanol were stirred in. On standing 0·78 g. of crystalline material separated. For convenience this is referred to in the present communication as fraction 4. It contained 1·8% ash ($CaSO_4$), was insoluble in glacial acetic acid, gave the colour test for tyrosine, and on drying *in vacuo* at 40° gave the ash-free analysis quoted in Table 5. The values for C, H, N and mol. wt. (by titration) come within the limits quoted by Dakin [1918] for his various samples of hydroxyglutamic acid. 0·4 g. of fraction 4 gave on treatment 0·27 g. of crystalline Cu aspartate, and the aspartic acid recovered from this was found to be almost completely racemized, the rotation in 10% HCl being $[\alpha]_D = +0·7°$ as against +25·6° given by the natural *l*(−)-amino acid. No glutamic acid hydrochloride could be separated from the mother liquor, which was not treated exhaustively, however, as our interest at the time was centred on the rotation of the isolated aspartic acid.

Protein material from metastases in liver of a woman with carcinoma of the colon. A fairly detailed analysis has already been described [Chibnall *et al.*, 1940]. The amino-acid solution recovered from the Ca salts precipitated in the 4th Foreman treatment was reduced to a small volume, when 0·396 g. crude tyrosine separated. The mother liquor on further reduction gave 0·4738 g. of crystalline material (fraction 5). This was insoluble in glacial acetic acid, gave a strong colour test for tyrosine, and on treatment gave 0·346 g. of Cu aspartate, the acid recovered from which was racemized; $[\alpha]_D = +4·5°$ in 10% HCl.

conditions for the isolation of his acid and his analytical figures approximate more closely to our fraction 4. We make no claim to have investigated the nature of our products at all exhaustively, for at the time they were isolated we had no such purpose in view, but we believe that sufficient evidence has been adduced to show that all of them must be complex mixtures containing varying amounts of tyrosine, dismutation products of cystine, aspartic acid, and in one case glutamic acid. We have ample evidence moreover that serine is precipitated as its Ca salt under the Foreman conditions, confirming the recent findings of Damodaran & Ramachandran [1941], who also showed that on hydrolysis of substances such as the phosphopeptone from casein (and hence of casein itself wherein the serine is present as phosphoserine) much deamination occurs with the formation of secondary acidic N-free decomposition products. As has been pointed out in an earlier section, the presence of serine will prevent the quantitative separation of aspartic acid as its Ca salt, and we know that the presence of the latter acid can retard the salting out of glutamic acid as its hydrochloride; hence we can legitimately infer that fractions 1 and 3, perhaps also one or more of the other three, contain larger amounts of these two dicarboxylic acids than we actually isolated by the simple procedure employed. In our dicarboxylic acid and base analysis of egg albumin, edestin and β-lactoglobulin [Chibnall *et al.*, 1943] the amount of unidentified N in the respective end fractions from the 2nd series of Foreman operations was very small (*ca.* 0·3% protein-N) and an exhaustive search has convinced us that these do not contain any aspartic acid, glutamic acid or unknown dicarboxylic acid. All these observations convince us that Dakin's products from casein, and also Calvery's [1931] product from egg albumin, were mixtures similar to those described above. Dakin's varying yields can almost certainly be correlated with the fact that he used glacial acetic acid to separate his new acid from residual amounts of glutamic and aspartic acids. Fraction 1 shows clearly that the solvent action of this reagent depends on the physical condition of the material being extracted, and if this be a fine amorphous powder much glutamic and aspartic acids can pass into solution.

Similar criticisms to those given above apply to the substance isolated from casein (1·5 kg.) in very small yield (20 mg.) by Gulland & Morris [1934]. These workers failed

Table 5. *Compositions of 'β-hydroxyglutamic acid' fractions*

Source	Fraction	C %	H %	N %	Organic-S %	Mol. wt. (by titration)
Egg albumin	(1)	31·46	6·10	9·02	4·53	—
	(2)	37·90	6·60	8·88	1·94	164
	(3)	33·33	5·58	6·70	5·20	163
Ox heart	(4)	36·03	5·91	8·84	1·28	168
Human liver	(5)	33·10	5·96	9·70	0·92	162
Hydroxyglutamic acid requires		36·79	5·56	8·59	—	163
Glutamic acid requires		40·80	6·17	9·50	—	147
Aspartic acid requires		36·08	5·31	10·53	—	133
Cysteic acid requires		21·30	4·14	8·28	18·95	169

The analyses given in Table 5 show that under varying conditions products having lower values for C, H and N than those of glutamic acid can be separated, and that if the assumption be made that these are dibasic acids their molecular weights computed by titration range from 162 to 168. Dakin, it will be recalled, used more standard

to separate any product by Dakin's procedure and investigated the N carried down in the $BaSO_4$ obtained on removal with baryta of the H_2SO_4 used in the hydrolysis. Their treatment was extensive and when much extraneous material had been separated they obtained a fraction, referred to above, which was rich in organic-S, showing that

they were dealing with dismutation products of cystine. At a later stage they claim to have isolated 20 mg. hydroxyglutamic acid after a preliminary phosphorylation with phosphoric acid and phosphoric oxide. It may be mentioned that no evidence was adduced to show that any such condensation took place, and the reagent employed may have done no more than aid the attenuation of what was evidently a complex mixture.

Appendix 3. Solubility of the Ca Salts under the Conditions of the Modified Foreman Procedure

Foreman [1914*a*] showed that when an aqueous solution of either aspartic or glutamic acid (1 g. in 20 ml.) was treated with lime and then with 9 vol. ethanol the Ca salt was quantitatively precipitated. Under the conditions of our original modified lime-ethanol procedure, however, all the amino-acids in the protein hydrolysate are present as hydrochlorides, and our early work showed that the $CaCl_2$ thereby introduced into the system raised the small residual solubility of the Ca dicarboxylates. Solubility factors were accordingly obtained from experiments in which the respective dicarboxylic acid hydrochlorides were passed through the procedure then in use, and these were employed as a guide in our researches until 1939, when our attention became focused more sharply on the problem by the claim of Kögl, Erxleben & Akkermann [1939] that the Ca salt of synthetic *dl*-glutamic acid (a mixture of the *l*(+)- and *d*(−)-antipodes) had 10 times the solubility of that of the natural *l*(+)-antipode, and that our procedure was accordingly unsuitable for the isolation of racemic glutamic acid from protein hydrolysates. We were able to refute these claims by appropriate experiments showing that the solubilities of the 2 salts were similar and by the demonstration that our procedure had already led to the isolation of relatively large amounts of partially racemized acid from hydrolysates of gliadin and edestin [Chibnall *et al.*, 1940; see also Graff, Rittenberg & Foster, 1940].

More detailed experiments with the Ca salts of glutamic and aspartic acids were then undertaken to ascertain the effect of the degree of racemization and the concentration of the salts concerned on solubility, and also of the concentration of $CaCl_2$ present. The technique employed was as follows. A weighed amount of the acid was dissolved in about 30 ml. of water and to this was added the necessary aliquot of a standard solution of $CaCl_2$, followed by an excess of CaO. The mixture was stirred for a few minutes and then filtered with suction through a Whatman no. 50 filter paper. The residue was washed repeatedly with small amounts of warm water, the collected filtrates made up to 100 ml. and 9 vol. of 95% ethanol added. The precipitate was filtered off after 10 min. (leaving for longer periods up to 24 hr. made no significant difference), washed with 95% ethanol, and the collected filtrates were taken to dryness *in vacuo*. The residue was then submitted to Kjeldahl analysis, using 20 ml. H_2SO_4; duplicate determinations tallied to 0·2 mg. N. Figs. 1–3 represent graphically the solubilities for various acid and $CaCl_2$ mixtures. Although somewhat meagre in some cases the data taken as a whole permit us to draw certain conclusions and can be put to limited use with protein hydrolysates.

The curves given in Fig. 1 show that the solubility of the Ca salt of *l*(+)-glutamic acid increases with the concentration of $CaCl_2$ and with the initial amount of glutamate. This latter phenomenon can possibly be explained in terms of complex ion formation between the Ca glutamate and $CaCl_2$, for there is evidence for the presence of a double salt of these 2 compounds in the Ca precipitate. The curves given in Fig. 2 suggest that in the presence of $CaCl_2$ the solubility of the *d*(−)-salt is slightly less than that of the natural antipode, and the same is true of the *dl*-salt made

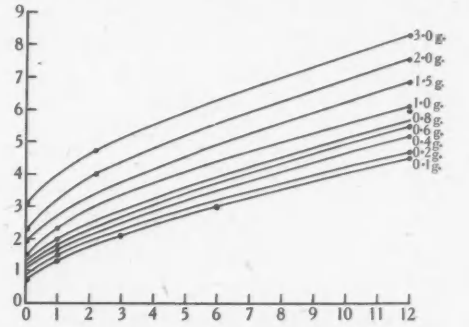

Fig. 1. Ordinates represent the residual solubility of the Ca salt of *l*(+)-glutamic acid when aqueous solutions containing various concentrations of the salt, and of $CaCl_2$, are treated with 9 vol. of 95% ethanol (mg. N per l. of aqueous-ethanol). The abscissae represent the concentration of $CaCl_2$ in the aqueous solution before addition of ethanol (g. per 100 ml.).

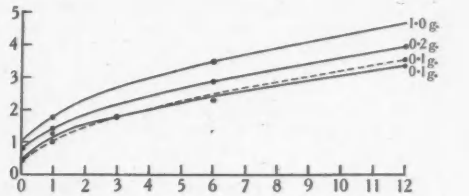

Fig. 2. Similar systems for the Ca salts of *d*(−)-glutamic acid (dotted-line curves) and of *dl*-glutamic acid (full-line curves).

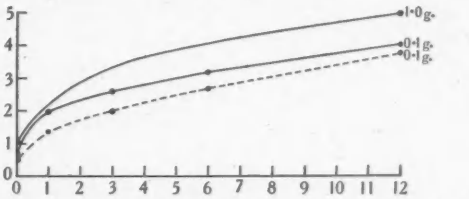

Fig. 3. Similar systems for the Ca salts of *d*(+)-aspartic acid (full-line curves) and of *dl*-aspartic acid (dotted-line curves).

from the synthetic acid, which Kögl *et al.* [1939] have shown to be a mixture of the 2 antipodes. The curves given in Fig. 3 show that similar conclusions apply to the Ca salts of aspartic acid.

From these findings it is possible to compute tentative solubility corrections for a dicarboxylic acid analysis covering no more than the first complete lime-ethanol treatment, provided that the concentration of HCl and of the aspartic or glutamic acid at each precipitation is known. Fortunately the necessary data are available for most of our recent analyses; and those for the glutamic acid of egg albumin are set out in Table 6, the assumption being made that the *l*(+)- and *dl*-salts have independent solubilities.

except that of the gliadin aspartic acid, and the low isolation value here may be due to the unsatisfactory procedure used to cover the various stages leading to the 2nd lime-ethanol treatment.

It is possible to calculate in the same way solubility corrections for the 2nd complete lime-ethanol treatment: these are very small and possible errors of no significance. In terms of the weight of protein the computed values for the above-mentioned proteins, in the order named, are 0·2, 0·13, 0·08% glutamic acid and 0·05, 0·03 and 0·03% aspartic acid respectively.

As in the case of the 3 particular proteins mentioned in Table 7 the computed values are in general slightly less than those found experimentally we have thought it safe to

Table 6. *Solubility corrections for the glutamic acid in egg albumin hydrolysates*

1st complete lime-ethanol treatment					Solubility correction		Wt. of glutamic acid (corrected)	
		Glutamic acid present						
Precipitation no.	Aqueous volume ml.	*l*(+)-acid g.	*dl*-acid g.	$CaCl_2$ present (g./100 ml.)	*l*(+)-acid (mg. N/100 ml.)	*dl*-acid (mg. N/100 ml.)	*l*(+)-acid g.	*dl*-acid g.
1	500	9·0	1·0	1·45	3·75	1·6	0·232	0·101
2	500	8·6	0·8	1·4	3·5	1·5	0·223	0·093
3	500	0·7	0·4	0·4	1·3	1·2	0·081	0·075
4	500	0·25	0·4	0·15	1·0	1·0	0·063	0·063
							0·599	0·332
Isolated during 2nd complete lime-ethanol treatment							0·721	0·60

The solubility corrections suggest amounts of the *l*(+)- and *dl*-acids that are less than those actually isolated during the 2nd complete lime-ethanol treatment [*vide* Chibnall *et al.*, 1943] indicating either that the solubilities had been enhanced by the presence of the other amino-acids in the hydrolysate or possibly that the isolation of the hydrochlorides from the end fraction of the first lime-ethanol treatment had not been quantitative. It is to be noted, however, that the crops of partially racemized glutamic acid isolated from this hydrolysate contain the true racemate (as opposed to the *dl* mixture) of Town [1941], since these give rise to a 3:5-dinitrobenzoyl derivative, m.p. 204° (private communication from Dr Town). The amounts isolated during the second treatment confirm our deduction that the solubility of the salt of the racemic acid is of the same order as that of the *l*(+)-antipode and refute the claim of Kögl *et al.* [1939] to which attention has already been drawn.

In terms of the weight of protein the results for edestin, egg albumin and gliadin are given in Table 7; as in no case have we examined the optical rotation of the aspartic acid the computed values are based on the solubility corrections for the Ca salts of the natural *d*(+)-acid. Considering the assumption made the agreement is reasonable in all cases apply similar corrections to such of our earlier dicarboxylic acid analyses, covering only the first complete lime-ethanol treatment, as we consider justified in light of experience.

Table 7. *Comparison of quantities of amino-acids isolated by 2nd lime-ethanol treatments with computed solubility corrections*

	Glutamic acid		Aspartic acid	
Protein	Computed from solubility corrections %	Isolated during 2nd complete lime-ethanol treatment %	Computed from solubility corrections %	Isolated during 2nd complete lime-ethanol treatment %
Edestin (3)	1·26	1·19	0·53	0·71
Egg albumin (2)	1·51	2·11	0·52	0·71
Gliadin	1·19	1·10	0·36	0·06

In the 2 casein analyses the optical rotation of the various crops of glutamic acid hydrochloride was determined, and solubility corrections for the amount of *dl*-acid present have been applied. We believe that the new values

Table 8. *Corrected aspartic and glutamic acid contents of some proteins*

	Glutamic acid			Aspartic acid		
Protein	From 1st complete lime-ethanol treatment %	Solubility correction %	Total %	From 1st complete lime-ethanol treatment %	Solubility correction %	Total %
Casein (1)	20·69	1·31	22·00	6·14	0·54	6·68
Casein (2)	20·67	1·33	22·00	6·10	0·58	6·68
Horse carboxyhaemoglobin	6·05	0·74	6·75	8·76	0·56	9·32
Cattle haemoglobin	5·84	0·52	6·36	8·03	0·56	8·56

for this protein are reasonably quantitative. In the 2 haemoglobin analyses the Cu_2O treatment was not applied and the glutamic acid was not examined, consequently we expect that all the new values will ultimately be shown to be too low.

Finally we should like to mention one point that has puzzled us for some years. Our own extensive experience with the lime-ethanol method of dicarboxylic acid analysis has shown that the first Ca precipitate carries down with it much extraneous N; yet Damodaran [1931], in his modification for determining the dicarboxylic acid-N of proteins on a micro-scale, found that the amount of N carried down was in reasonable agreement with that suggested by the older analyses for the dicarboxylic acid contents of the protein concerned. Our solubility data represented graphically in Fig. 1–3 suggest an explanation of the anomaly. Damodaran hydrolysed the protein with HCl and removed the excess of mineral acid by evaporation to a syrup *in vacuo*, as in a Van Slyke analysis. We find that under these conditions much HCl in excess of that bound as hydrochloride is retained, and to remove this excess it is necessary to add water and again evaporate, repeating the operation 4 or 5 times. At the precipitation stage, therefore, Damodaran's hydrolysate must have contained an excess of $CaCl_2$, which increased the residual solubility of the Ca dicarboxylates and reduced the amount of N precipitated to a value that showed fortuitous agreement with the dicarboxylic acid-N calculated from earlier analyses.

SUMMARY

1. The lime-ethanol method of Foreman for the determination of aspartic and glutamic acids in protein hydrolysates, and the various modifications of it introduced by other workers, have been investigated in considerable detail.

2. During treatment with the lime the cystine present in the hydrolysate undergoes partial dismutation to the sulphinic or sulphonic acids; these are precipitated with the Ca dicarboxylates by the ethanol, together with small amounts of cystine itself, tyrosine, serine and bases.

3. The Ca salts of these cystine dismutation products are very insoluble and interfere with the estimation of aspartic acid as Cu aspartate. To prevent their formation it has been found advantageous to remove all cystine as the cuprous mercaptide of cysteine before the hydrolysate is made alkaline with lime.

4. The solubility of the Ca dicarboxylates under the conditions of the modified Foreman procedure we have used has been investigated by clarifying the lime-ethanol filtrate, removing the bases and a major part of the monoamino-acids, and repeating the lime-ethanol treatment on the final mother liquor. Significant amounts of both aspartic and glutamic acids have been isolated.

5. Based on these findings a new extended procedure has been elaborated which there is reason to believe has given values for the aspartic acid and glutamic acid contents of gliadin, edestin and egg albumin that are accurate to about 2%.

6. The possibility of applying solubility corrections to the dicarboxylic acid results obtained in an analysis covering only one complete lime-ethanol treatment has been investigated. Results for casein, horse haemoglobin and cattle haemoglobin obtained in this way are higher than those hitherto recorded, and it is probable that those for the 2 latter proteins are still too low.

7. Reasons are given for the belief that the 'hydroxyglutamic acid' fractions obtained by earlier workers are complex mixtures containing *inter alia* aspartic acid and dismutation products of cystine.

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The Dicarboxylic and Basic Amino-acids of Edestin, Egg Albumin and β-Lactoglobulin

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The various investigations made in this laboratory during the few years preceding 1938 on the amino-acid composition of leaf proteins have been briefly summarized elsewhere [Chibnall, Rees & Williams, 1943]. These served to convince us that many of the methods of analysis we were then using, in particular those for the dicarboxylic acids and the bases, could not be applied to better characterized proteins with the precision demanded by the Bergmann-Niemann generalizations on protein structure [Chibnall, 1942] until their respective advantages and limitations had been more clearly defined and incidental working losses drastically reduced.

In accordance with this view we have thoroughly explored the Foreman method for the separation of the dicarboxylic acids, and an account has already been given [Bailey, Chibnall, Rees & Williams, 1943] of the various experiences which led us ultimately to devise a satisfactory procedure. This is incorporated in the general scheme of analysis for the dicarboxylic and basic amino-acids which is described in the present paper. Our aim has been to isolate these two groups of amino-acids as pure derivatives and in quantitative yield by step-wise removal without overall loss, and to overcome the solubility factors inherent in all precipitation methods it was necessary to remove also, at the appropriate stages, a large part of the monoamino-acids and proline. In formulating a procedure for doing this we have been guided to a large extent by our experience of the butanol method of Dakin [1918; 1921; 1923] and of the Cu salt method of Town [1928; 1936; 1941]. To anyone who has had experience of the earlier Fischer methods of esterification and distillation the elegance of the Dakin procedure needs no emphasis, for the monoamino-acids are obtained in the convenient form of a white crystalline powder free from both proline and hydroxyproline. In this laboratory Dakin's method has been applied to myosin [Sharp, 1939] and to cocksfoot protein [Lugg; *vide* Chibnall, 1939, p. 162], the esters subsequently being distilled under low pressure through a modern fractionating column. A certain degree of success was obtained, but the method was not proceeded with for two reasons. In the first place we found that small amounts of the dicarboxylic acids and bases were extracted by the butanol. Secondly, we found that extensive diketopiperazine formation occurred, even when the butanol extraction was carried out under reduced pressure, leading to losses amounting to several per cent of the total protein-N. The meagre data quoted by Dakin [1923] in his analysis of zein suggest that much of the N that he was unable to account for was located in the butanol-proline fraction. Our experience of the Cu salt separation has been the same as that of Town himself, in that we have found that the solubility of one particular salt or group of salts can be profoundly influenced by the presence of others. The method must therefore be used with discrimination, and unless circumstances warrant, no exaggerated idea of the purity of the products isolated must be entertained. The procedure for the estimation of the dicarboxylic acids and the bases given below embodies our cumulative experience of these two methods and it leads, incidentally, to the separation of the monoamino-acids in excellent yield but in several fractions, each of which is a mixture. These could be used, either singly or collectively, for the estimation of the indi-

vidual components by the ester method, as had been our original intention, but the new partition chromatographic method of Gordon, Martin & Synge [1941; 1943], which is applied to the hydrolysate without any preliminary fractionation, has proved so strikingly successful that it is doubtful if our products will be required for this purpose.

EXPERIMENTAL

1. *Scheme of analysis*

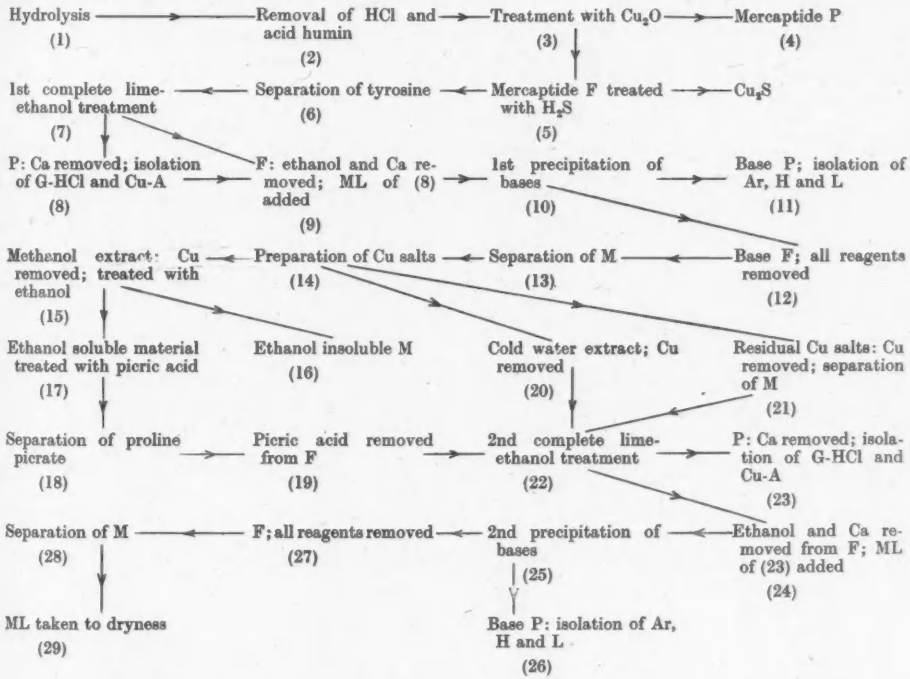
The cardinal point in the procedure is that no reagent may be introduced into the main hydrolysate unless it can be quantitatively removed at a later stage without appreciable loss of N, for in an extended scheme such as we have adopted the working losses are cumulative and can soon amount to several per cent of the total protein-N. It follows that certain reagents either cannot be used at all or only at a stage when there is reason to believe they comply with this provision.

Now it has been the experience of all protein analysts that if H_2SO_4 be used to effect hydrolysis and the mineral acid is subsequently removed with baryta, the resulting $BaSO_4$ obstinately retains N much in excess of that due to humin. We find nevertheless that after the removal of the cystine and the major part of the aspartic acid, i.e. at stage 9, Ba^{++} or SO_4^{--} can be introduced into the hydrolysate and removed in the usual way as $BaSO_4$ without appreciable loss of N, an observation which points strongly to the fact that the excess N carried down when this latter operation is conducted at an earlier stage represents these two amino-acids, very probably in the form of double salts with $BaSO_4$. Dakin [1921] mentioned the difficulty of completely removing Ba^{++} with H_2SO_4 from solutions of Ba hydroxyaspartate, but we are unaware of any corresponding citation for the two above-mentioned amino-acids. To avoid these large losses certain workers have preferred to hydrolyse the protein with HCl, remove the excess acid in the usual way

Diagram 1.

Scheme of amino-acid analysis.

Abbreviations: G-HCl, glutamic acid hydrochloride; Cu-A, Cu aspartate; Ar, arginine; H, histidine; L, lysine; M, mono-amino-acids; P, precipitate; F, filtrate; ML, mother liquor.


Hydrolysis (1)
Removal of HCl and acid humin (2)
Treatment with Cu_2O (3)
Mercaptide P (4)
1st complete lime-ethanol treatment (7)
Separation of tyrosine (6)
Mercaptide F treated with H_2S (5)
Cu_2S
P: Ca removed; isolation of G-HCl and Cu-A (8)
F: ethanol and Ca removed; ML of (8) added (9)
1st precipitation of bases (10)
Base P; isolation of Ar, H and L (11)
Methanol extract: Cu removed; treated with ethanol (15)
Preparation of Cu salts (14)
Separation of M (13)
Base F; all reagents removed (12)
Ethanol soluble material treated with picric acid (17)
Ethanol insoluble M (16)
Cold water extract; Cu removed (20)
Residual Cu salts: Cu removed; separation of M (21)
Separation of proline picrate (18)
Picric acid removed from F (19)
2nd complete lime-ethanol treatment (22)
P: Ca removed; isolation of G-HCl and Cu-A (23)
Separation of M (28)
F; all reagents removed (27)
2nd precipitation of bases (25)
Ethanol and Ca removed from F; ML of (23) added (24)
ML taken to dryness (29)
Base P: isolation of Ar, H and L (26)

by evaporation *in vacuo* and then free the hydrolysate from Cl^- with Ag_2O. The precipitate of AgCl is much less bulky than the corresponding one of $BaSO_4$, and less excess N is retained, but in our experience it is still appreciable and may amount in some cases to as much as 4% of the total protein-N. Such losses are due to the insolubility of the Ag mercaptide of cysteine, even at fairly high concentrations of H^+ [Bailey, private communication], and of both Ag aspartate and glutamate, for at stage 9 Ag^+ or Cl^- can be removed as AgCl at *p*H 3 without loss of N. According to our experience CuS, Cu_2S and Ca oxalate do not retain N (except humin-N, if present); Cu^+, Cu^{++} and Ca^{++} therefore can be removed from the hydrolysate at any stage as these salts respectively. Lastly, if phosphotungstic acid be used to precipitate the bases, this acid can be removed from both precipitate and filtrate in the usual way with amyl alcohol-ether mixture without loss of N, provided that the aqueous phase be first saturated with ether and ether-water be used for all aqueous washings. If this precaution is not adopted the numerous aqueous washings soon reduce the ether content of the organic solvent mixture to such a low level that a small amount of N is extracted by the amyl alcohol on the Dakin principle.

All of the above-mentioned observations have been taken into account in devising the scheme of analysis set out in Diagram 1. The rationale is as follows. The protein is hydrolysed with HCl, the excess acid and the acid humin removed in the usual way and the clarified solution treated with Cu_2O (3) to precipitate the residual humin and the cystine as the cuprous mercaptide of cysteine. Following the separation of some crude tyrosine (6) the hydrolysate is passed through the first complete lime-ethanol treatment for the isolation of the dicarboxylic acids (7–8). The mother liquor from this is joined with the Ca-free filtrate (9) and the first precipitation of the bases is made with phosphotungstic acid (10); cystine and the major part of the aspartic acid having been previously removed the precipitate (11), according to our experience, consists almost exclusively of basic material. All reagents and the residual Cl^- are next removed from the base filtrate, which is then reduced to a small volume. On standing, a partial separation of the less soluble monoamino-acids (leucine, phenylalanine, tyrosine and methionine) occurs (13). The soluble products are then passed through the Cu salt treatment of Town, when additional amounts of the above-mentioned mixed amino-acids (21), a valine-*iso*leucine fraction (16) and a large part of the proline (18) are separated. These last few stages serve to reduce the monoamino- and imino-acid content of the hydrolysate to between one-third and one-fourth of that present at stage 7, and it is now ready for the 2nd complete lime-ethanol treatment (22). Following this a 2nd precipitation of the bases is made with phosphotungstic acid (25), and as the major part of the proline has been previously removed the precipitate (26) contains very little non-basic-N. All reagents are then removed from the base filtrate and the clarified solution reduced to a small volume, when further crops of monoamino-acids separate (28). The ultimate filtrate is taken to dryness *in vacuo* and granulated with acetone to give a fine white non-hygroscopic powder (29).

It will be seen that this extended procedure has enabled us to make a direct investigation, via stages 22 and 25, of the solubility factors operative during the first treatment for the dicarboxylic acids at stage 7, and the first treatment for the bases at stage 10 respectively. This is, of course, one of the essential features of the analysis, and as we determine the amide-N at stage 2 and the composition of the mercaptide precipitate at stage 4 we have knowledge of the gross weight and total N of the hydrolysis products present in all the various fractions. We are thus able not only to compute the overall losses and indicate from flow sheets at which stages these occur, but we can determine directly the gross weight of products given on hydrolysis of 100 g. of protein, a factor that can also be computed indirectly in terms of the total N and of that in non-peptide form [see Chibnall, 1942].

Needless to say all reagents employed must conform to certain standards of purity. $CuCO_3$ must be free from sulphate, chloride, and other metals; the commercial samples we have tested do not satisfy these requirements and we have prepared our own from A.R. $CuSO_4$. H_2SO_4 must be N-free. 12-Phosphotungstic acid must be not only N-free but also Na-free; few commercial products satisfy this latter requirement. The samples that we have used were not purified through ether by Winterstein's [1898] method as we were not specifically concerned with phosphotungstate solubilities [Van Slyke, Hiller & Dillon, 1942]. Ag_2O and Cu_2O must be washed free from salts and alkali, $Ca(OH)_2$ must be free from sulphate and iron; we have used a commercial sample but the preparation from Ca is recommended.

2. *Analysis of edestin*

An estimation of total N was made at every convenient stage in the analysis [Chibnall *et al.*, 1943] and flow sheets giving details of aliquots withdrawn were kept throughout so that factors were available to correct all values to the weight of protein (W/P) or to the total protein-N (T.P.N.) as the case may be. The numbers at the head of each section given below refer to the stage numbers in Diagram 1.

Details of analysis

1, 2. 61·741 g. of moisture- and ash-free protein, containing 11·52 g. of N or 18·65% were hydrolysed for 23 hr. with 400 ml. 20% HCl. The excess HCl was removed by evaporation *in vacuo* to a gum; water was then added and the mixture again reduced to a gum, the latter operation being repeated 6 times in all. Experience has shown that under these conditions all excess HCl is removed, and the amount present is no more than that required to bind the amino-acids and ammonia. Ammonia-N, obtained by distillation of an aliquot with alkali at *p*H 12·5 in a micro-Kjeldahl apparatus, was 10·42% T.P.N. This value is, of course, much in excess of the true amide-N, which is 9·49% [Gordon *et al.*, 1941; Chibnall, 1942]. The acid humin was too small to be separated, and it was removed later with the alkali humin in the mercaptide precipitate at stage (3). Data for the composition of the humin (Table 1) were obtained from a sample made by very cautiously treating an aliquot of the solution with lime to *p*H 7·5. Maximum precipitation was not obtained at *p*H 7 with NaOH. The separated humin was washed and dried; N and ash ($CaSO_4$) were determined.

3, 4. The main solution was treated with Cu_2O as described in a previous paper [Bailey *et al.*, 1943]. The Cu^+-free filtrate was colourless, showing that all humin had been removed. The precipitate contained S≡67·3% total protein-S and N≡2·20% T.P.N. The S is in excess of that due to cystine and must represent methionine. Colorimetric estimation before and after the precipitation showed that the precipitate contained by difference tyrosine N≡0·41% T.P.N. These two latter amino-acids and probably also very small amounts of phenylalanine and leucine must have separated, as such or as copper complexes, from the hydrolysate during the period when the mercaptide mixture was allowed to stand at 10° overnight, for the removal of Cl^- as Cu_2Cl_2 had reduced the acidity to about *p*H 3·5, in which region the solubility of these amino-acids is much depressed [Hill & Robson, 1934; Baptist & Robson, 1940].

Table 1. *Composition of the organic material in the mercaptide precipitate (edestin)*

	Wt. g.	Wt. as % of protein	N g.	N as % total protein-N
Humin	0·729	1·16	0·0759	0·61
Cystine (0·2255 g. S)	0·846	1·37	0·0985	0·85
Methionine (0·1385 g. S)	0·644	1·04	0·0605	0·53
Tyrosine	0·247	0·4	0·0191	0·21
Total	2·466	3·97	0·254	2·20

5, 6. The mercaptide filtrate and washings were collected, a few ml. HCl added and Cu^+ removed with H_2S. As the washing of inorganic precipitates such as Cu_2S must be thorough if working losses are to be kept down to the minimum, the details of our method are given fairly fully. The many precipitates of CuS obtained in later stages were treated in a similar way.

The solution is transferred to a Büchner flask fitted with a rubber bung carrying a glass tube, for the entry of H_2S, which reaches nearly to the bottom of the flask, the side limb being closed with a short length of pressure tubing and a screw clip. H_2S is then passed in under a pressure of about 18 in. water and the contents of the flask shaken until the Cu_2S coagulates to leave a clear liquid and no more gas is taken up. The mixture is then filtered on a Büchner funnel, using a hardened paper, and before allowing the precipitate to pack hard it is washed 2 or 3 times with cold water (stirring). Meanwhile the main filtrate is being concentrated *in vacuo* to a syrup to remove H_2S and excess HCl if this be present. The precipitate is removed from the filter paper, transferred to a 250 ml. centrifuge bottle fitted with a rubber bung, shaken vigorously with cold water until it is completely disintegrated and again filtered. This operation is generally repeated twice. The precipitate is next transferred to a 1 l. Pyrex beaker, 300–400 ml. of water are added and the beaker immersed in a boiling water-bath for 30–45 min. The precipitate is then filtered off and the operation repeated 5 or 6 times. Occasionally the washings become colloidal and it is necessary to add a few drops of mineral acid to induce flocculation. The collected washings are then reduced *in vacuo* to a syrup and joined with the main filtrate. The resulting solution is then tested for traces of Cu^+ by passing in H_2S, filtered if necessary from any sulphide or elementary S present, and made up to standard volume for determination of N.

The flow sheets showed that at the end of stage 5 there was no detectable loss of N. The remainder of the solution was treated with lime to *p*H 3·5 and reduced to 200 ml. On standing 4 days at 0° a small crop of crude tyrosine separated; colorimetric estimation showed that 86% of the product was tyrosine [Meyer, Field & Klemm, 1940].

7. The filtrate and washings from the tyrosine, of volume about 1200 ml., were treated with freshly prepared cream of lime until an excess remained undissolved. The mixture was then reduced *in vacuo* to a syrup to remove ammonia. Water was then added to make the volume 400 ml., followed by 3600 ml. of 95% ethanol, which were added quickly with vigorous stirring. The separated Ca salts and excess lime were filtered off without delay on a Büchner funnel, using a hardened filter paper, and the extraneous mother liquor removed by pressing the mass to a hard pad, which was then washed with a little absolute ethanol. Suction was continued until most of the ethanol passed through and the material could be reduced to a friable powder by rubbing with a spatula. Unless this precaution be adopted difficulty will be experienced, on account of the presence of ethanol, in redissolving the Ca salts in a small volume of warm water; the dry salts nevertheless are hygroscopic and the suction must not be continued too long, especially if the laboratory air is somewhat humid. The material was next transferred to a beaker, warm water added with stirring to dissolve the Ca salts, and without removing the residual excess lime the volume was adjusted to 400 ml.; 3600 ml. of 95% ethanol were then added.

The Ca salts were filtered off, washed as before and extracted with about 800 ml. warm water, the small residue of lime being washed 8 times by stirring with 150 ml. water kept near the boil for a few minutes and centrifuging. The clear liquors were collected, Ca^{++} removed as described below with oxalic acid and the mixture centrifuged. The clarified solution was reduced *in vacuo* to 200 ml. and reserved. The Ca oxalate was washed once with cold water, and then 8 times with 150 ml. hot water, the collected washings were cooled to room temperature and kept for some time to permit the recrystallization of the small amount of redissolved oxalate. The latter was removed by centrifuging,

the clear washings collected and reduced *in vacuo* to 50 ml. The small amount of oxalate that separated was centrifuged off and the clarified liquor added to the main solution mentioned above. The volume was then made up to 1 l. and 5 ml. withdrawn for analysis. The total N present (corrected) was equivalent to 20·1% T.P.N.

Without delay each of the two lime-ethanol filtrates was reduced *in vacuo* to a syrup to remove ethanol and traces of NH_3. The syrup from the first filtrate was then taken up in about 1 l. of water and oxalic acid added until no further precipitation occurred. The Ca oxalate was removed by centrifuging, washed once with hot water and reserved for treatment later. The clear centrifugate was taken to a syrup *in vacuo*, water added and again removed *in vacuo*, this treatment being applied 4 times in all to remove HCl set free from any $CaCl_2$ that had originally been present in solution. The removal of HCl at this stage is necessary, as Ca cannot be completely precipitated as oxalate in its presence. The syrup was again taken up in water and just sufficient oxalic acid added to throw out a small precipitate of Ca oxalate, which was removed by centrifuging and washed once. Both oxalate and washing were reserved. The syrup from the 2nd lime-ethanol filtrate was treated with oxalic acid, the Ca oxalate washed once as before and reserved. The clear centrifugate was reduced *in vacuo* 4 times as before to remove free HCl. Just sufficient oxalic acid was then added to throw out a fairly heavy precipitate of Ca oxalate, which was washed once and reserved. Free HCl was again removed as before, followed by precipitation of the Ca as oxalate. The number of times this operation must be repeated varies with the solution concerned; in the present case 4 treatments were necessary. All the precipitates of oxalate from both filtrates were then collected and washed thoroughly 8 times in succession with 250 ml. boiling water. The combined filtrates, including those from the initial 1st washings, were then reduced *in vacuo*, added to the two main centrifugates and the solution reduced *in vacuo* to 200 ml. Final adjustments for the removal of Ca and oxalate ions were then made, the small precipitate washed and the filtrate and washings made up to 1 l. for the determination of total N.

8. (Abbreviations: G-HCl, glutamic acid hydrochloride; Cu-A, Cu aspartate.) The solution was reduced *in vacuo* to 200 ml., the very small amount of Ca oxalate that separated was filtered off, the filtrate and washing again reduced *in vacuo* to 50 ml., filtered from a further small amount of separated Ca oxalate, and the filtrate and washings transferred to an open dish with about 30 ml. conc. HCl. Evaporation was then allowed to proceed to incipient crystallization on a water-bath. After standing at room temperature overnight the separated material was filtered off on a sintered glass crucible, washed with a little conc. HCl followed by absolute ethanol, and dried off *in vacuo* over NaOH to give G-HCl crop 1.

The filtrate was reduced *in vacuo* 6 times to remove free HCl and taken up in 600 ml. boiling water. Purified $CuCO_3$ was added in excess and the hot solution filtered immediately on a hot funnel to remove the excess carbonate. The filtrate was cooled and kept at 0° for 4 hr. Cu-A crop 1 was filtered off and washed many times with water, followed by ethanol and ether. Cu^{++} was removed from the filtrate with H_2S in the presence of HCl, and the CuS washed. The clarified filtrate and washings were collected, reduced *in vacuo* to 20 ml., transferred to an evaporating basin, a few ml. conc. HCl added and the mixture reduced to 5–6 ml. on a water-bath. On standing for 20 hr. at 0° G-HCl crop 2 separated; this was filtered, washed and dried as described above.

On further treatment in the same way Cu-A crop 2 and G-HCl crop 3 were obtained. Excess HCl was removed from the mother liquor and a 3rd lime-ethanol precipitation made from an aqueous volume of 30 ml. The filtrate was freed from Ca^{++} as described above. The precipitate was redissolved in water and Ca^{++} removed with oxalic acid until the *p*H was reduced to 3·2. The clarified solution was next reduced to about 20 ml. and kept at 0° overnight when a small crop of crude tyrosine, ≡0·30% w/P, separated. Ca^{++} was then removed from the mother liquor. The clarified solution gave Cu-A crop 3 but no glutamic acid. The lime-ethanol treatment was then applied twice in succession, from aqueous volumes of 10 and 30 ml. respectively. The clarified precipitate, on standing many weeks, gave G-HCl crop 4 containing 4% ash ($CaSO_4$), but no Cu aspartate could be separated. The final mother liquor contained 0·1173 g. N (corrected), ≡1% T.P.N. Flow sheets showed that at the end of stage 8 the overall working losses amounted to 25·8 mg. N (actual) or 30·4 mg. N (corrected), equivalent to only 0·27% T.P.N.

9, 10. The five lime-ethanol filtrates (the last two still contained Ca^{++}), together with the mother liquor mentioned above, were collected, freed from ethanol, and made up to 1 l. The total N, uncorrected, was 7·82 g. For the precipitation with 12-phosphotungstic acid (P.T.A.) two trial runs with a mixture of 50 ml. solution, 50 ml. water and 14 ml. conc. HCl showed that 20 g. P.T.A. in 20 ml. water were in slight excess. 895 ml. of the main solution were accordingly diluted to 1795 ml. with water and 240 ml. conc. HCl added. The whole was warmed to 60° and a warm solution of precipitant (216 g. P.T.A. in 400 ml. water) poured in with stirring. The two trial runs were then added and the mixture allowed to stand for 2 days at room temperature. The precipitate was filtered on a Büchner funnel using a hardened paper, suction being maintained until the material had been pressed down to a hard pad and no further liquid passed. The material, still on the paper, was next puddled to a fine cream with 100 ml. of wash solution (2·5 g. P.T.A. in 100 ml. of *N* HCl); suction was then applied and the material again pressed down to a hard pad. This pad was broken with a spatula and transferred to a cylindrical separating funnel. The Büchner funnel and hardened paper were then well washed with water, the washings being transferred to the separating funnel to give a final aqueous volume of about 1500 ml. 100 ml. conc. HCl were then added and the mixture shaken vigorously to give a fine cream. To remove the P.T.A. the following organic solvent was used: amyl alcohol (pyridine-free and well washed with approximately *N* HCl), 20 parts; 0·720 ether, 25 parts; absolute ethanol, 1 part. 200 ml. of this were added to the funnel and the mixture shaken; a further 2 lots of 100 ml., making 400 ml. in all, were required to effect a clean separation. The solvent was drawn off and washed with 400 ml. of ether-water. This aqueous washing was added to the main aqueous solution, which was then washed with 4 lots of 50 ml. and 5 of 80 ml. of the organic solvent successively. The aqueous solution was then reduced *in vacuo* to a syrup to remove excess HCl. All the organic solvent solutions were then collected (vol. about 900 ml.), 600 ml. of ether added (to depress solubility of amino-acids) and the mixture washed 10 times successively with 60–70 ml. *N* HCl saturated with ether. The aqueous washings were collected (vol. about 1·5 l.) and washed 8 times successively with 60 ml. of the organic solvent. The latter were collected

(vol. about 450 ml.), 150 ml. ether added and the mixture washed 8 times successively with 70 ml. of the *N* HCl-ether-water. The collected aqueous washings were given two final washings with 40 ml. of the organic solvent and then reduced *in vacuo* to a syrup to remove excess HCl. This syrup, together with that from the main solution, was taken up in about 300 ml. water, filtered to remove a faint mist of amyl alcohol and the clear but slightly brown filtrate made up to 1 l. The total N was 3·894 g.≡33·8% T.P.N. The P.T.A. organic solvent solutions were reduced in volume to about 400 ml. and made up to 500 ml.; the total N was 6·7 mg.

11. The analysis of the base solution is described in Section 5.

12. The base filtrate was freed from P.T.A. by a procedure similar to that described above. The organic liquors retained only 6·0 mg. N. The clarified filtrate contained N≡34·9% and amino-N≡31·0% T.P.N. respectively. The procedure for the removal of inorganic ions was as follows.

Chloride. The excess HCl was removed by evaporation *in vacuo* to a gum, water being added and the operation repeated 6 times in all. The residue was taken up in water (300 ml.) and the bound HCl removed with moist Ag_2O, the solution being kept below *p*H 3 by the cautious addition of 0·5*N* H_2SO_4, of which about 300 ml. were required. The AgCl was filtered off on a Büchner funnel, transferred to a beaker, brought to the boil with 700 ml. of water (stirring) and again filtered. The washing was twice repeated. The collected washings were reduced *in vacuo* to 150 ml. and allowed to stand; $CaSO_4$ separated and was filtered off. A small amount of $CaSO_4$ had also separated from the main solution on standing; this was also filtered off and joined to that mentioned above. The combined material was then washed 3 times with hot water and the washed $CaSO_4$ reserved. The AgCl was next washed 8 times in succession by bringing to the boil with about 300 ml. of *N* HCl and filtering. The washings were taken to dryness *in vacuo* to remove excess HCl, precipitated with Ag_2SO_4, the precipitate washed and the filtrate added to the main solution.

Silver. The solution and washings were treated with H_2S, the Ag_2S filtered off on a Büchner funnel and washed in the same way as the Cu_2S described above.

Calcium sulphate. The solution and washings were concentrated to the incipient crystallization of $CaSO_4$; 2 vol. of 95% ethanol were then added and the mixture allowed to stand overnight. The $CaSO_4$ was filtered off and washed 5 times successively by boiling with 100 ml. of water for 30 min. The washings were concentrated to about 60 ml. *in vacuo*, allowed to stand for a few hours, the small amount of separated $CaSO_4$ filtered off and washed 3 times with hot water.

Sulphate. The solution and washings were collected and sulphate precipitated as completely as possible with baryta, the mixture being heated on a water-bath for about 1 hr. The precipitate was filtered off on a Büchner funnel and washed 6 times successively by bringing to the boil with about 200 ml. of water. The filtrate and washings (at *p*H 7·3) were collected, reduced *in vacuo* to about 200 ml. and without removing the small amount of separated $BaSO_4$ a slight excess of Ba^{++} was carefully removed with H_2SO_4. The precipitate was filtered off and washed. A second and final adjustment for Ba^{++} and SO_4^{--} was made after concentration of the filtrate and washings to a small volume.

The $BaSO_4$ and the AgCl from all these operations were collected and treated with 600 ml. *N* HCl at 80° for 8 hr. The filtrate and washings were collected, excess HCl and Cl^- removed in the usual way and the final filtrate added to the main solution. The working loss during the removal of all the above-mentioned inorganic ions was 17 mg. N actual and 23 mg. N corrected.

13, 14. In the edestin analysis stage 13 was omitted, and the solution of amino-acids was treated with $CuCO_3$ to make the Cu salts by the method of Town. These were granulated with A.R. acetone.

15, 16. (Abbreviation: M.A., monoamino-acids.) The dry salts, in a 250 ml. centrifuge bottle, were given 7 successive extractions with approximately 150 ml. carefully dried methanol (dehydrated twice over Ca) by shaking in a mechanical shaker for approximately 1 hr. each time. The insoluble residue was extracted repeatedly at room temperature with water until the extracts were colourless. These aqueous extracts were evaporated *in vacuo* and granulated with acetone as before. The dry material was then given 8 extractions as before with methanol. The residue from this last operation was taken up in water, evaporated to a syrup, and after granulation with acetone the extraction with methanol was continued, the 4th and 5th extracts being nearly colourless. The collected alcoholic extracts were reduced to a syrup, which was granulated with acetone and then re-extracted with the solvent. The alcoholic extracts were united and reduced to a syrup, which was then taken up in water and Cu^{++} removed with H_2S. After washing the CuS in the usual way the solution was reduced to 40 ml., and cooled to 0° overnight. The crystalline material that separated was filtered off, the mother liquor was reduced in volume, ethanol added to faint turbidity and the mixture warmed until the solution cleared. On standing at 0° overnight a 2nd crop of crystalline material separated. The collected products, M.A. crop 1, weighed 3·959 g. (5·318 g. corrected) and contained 11·74% N.

17, 18, 19. When present in fair concentration proline is partially precipitated by P.T.A. under the conditions of a base analysis; it was decided therefore to remove as much as possible of this imino-acid to prevent contamination of the second base precipitate at stage 25. The filtrate from the separation of M.A. crop 1 at stage 15 contained 0·165 g. amino-N and 0·204 g. non-amino-N (corrected 0·2216 and 0·2740 g. respectively). A small aliquot was first passed through the laborious $CdCl_2$ procedure of Town [1936], but the incidental losses were appreciable and the amount of non-amino-N in the final solution less than expected. Town himself found that the proline was not quantitatively precipitated by one treatment, and King [1941] records a similar finding. The remainder of the solution was therefore reduced to 30 ml. and picric acid (5·016 g.≡non-amino-N) in a small volume of hot water added. On standing at 0° a 1st crop of proline picrate (3·296 g.) separated and on reducing the volume a 2nd crop (1·153 g.) was obtained. The combined crops were equivalent to 1·996 g. proline (corrected). The mother liquor was acidified with HCl, the precipitated picric acid filtered off, the liquors extracted 6 times with ether to complete the removal, and reserved for stage 22.

20. Cu^{++} was removed from the solution of water-soluble salts, which was then reduced *in vacuo* to 25 ml. and kept overnight at 0°. M.A. crop 2 separated; wt. 1·9213 g. or 2·575 g. corrected; N=10·11%, all in the amino-form. The mother liquor was reserved for stage 22.

21. The residual Cu salts were extracted repeatedly with large volumes of water in a beaker heated on the water-bath, conditions which would remove any Cu aspartate. Each extract was filtered off, and while still hot Cu^{++} was

removed with H_2S. The CuS was washed in the usual way and the collected liquors reduced *in vacuo*. Three crops of monoamino-acids were obtained; collected these gave M.A. crop 3; wt. 7·3916 g. (corrected); N = 11·26%. The mother liquor was reserved for stage 22. The final insoluble residue, which was thought at the time to be excess $CuCO_3$, almost certainly contained, according to later experience, the very insoluble Cu salt of methionine and possibly also that of phenylalanine; it also contained subordinate amounts of humin, and these products are in large part responsible for the recorded loss of N during the Cu salt treatment (stages 15–20), which amounted to 0·209 g. (corrected) ≡ 1·94% T.P.N. To guard against the possibility that the 3 crops of monoamino-acids separated at stages 16, 20 and 21 respectively contained either aspartic or glutamic acid, though none gave any significant titration for dicarboxylic acids, an aliquot of each crop was taken, dissolved in hot water, NH_3 added to *p*H 8·8 and the mixture cooled [Osborne & Liddle, 1910*a*]. The crystalline mass of monoamino-acids was filtered off and the mother liquor reduced to a small volume. The lime-ethanol treatment was then applied, but the precipitate was free from N.

22, 23. (Abbreviations: G-HCl, glutamic acid hydrochloride; Cu-A, Cu aspartate.) The collected solutions were treated with a 1/10 vol. conc. HCl and boiled gently for 2 hr. to open the pyrrolidone ring. The subsequent treatment was similar to that applied at stages 7 and 8 and calls for no special comment. The lime-ethanol treatment was applied 3 times in all, from aqueous volumes of 60, 20 and 100 ml. respectively. The clarified solution from the 1st precipitate contained 0·1935 g. N (corrected 0·2599 ≡ 2·22% T.P.N.) and 0·1538 g. amino-N; from it was isolated 0·5457 g. Cu-A and 0·4578 g. G-HCl. The 2nd treatment gave 0·1148 g. Cu-A and in 2 small crops 0·1609 g. G-HCl; the 3rd treatment 0·0848 g. G-HCl. The various lots were collected to give 0·9102 g. (corrected) of Cu-A (Table 2, crop 4) and 0·9684 g. (corrected) of G-HCl (Table 2, crop 5); the latter contained 4·96% of $CaSO_4$. The final mother liquor contained 0·0270 g. N (corrected), equivalent to only 0·27% T.P.N.

24, 25, 26. The procedure followed that of stages 10 and 11, the base precipitation being made from a total volume of 290 ml. with 25 g. P.T.A. in 20 ml. water. The base solution contained 0·1185 g. N of which 0·0778 g. was amino-N; the total N (corrected) was 0·1592 g. or 1·29% T.P.N. The analysis for the 3 bases is given in Section 5; only 0·051 g. N, all in the amino-form and equivalent to 0·45% T.P.N., was unaccounted for. The separation of proline at stage 18 had therefore been effective.

27, 28, 29. The base filtrate was freed from P.T.A. and all inorganic ions as in stages 10 and 12. The final solution was evaporated to a small volume and taken to dryness in a vacuum desiccator. The residue was a non-hygroscopic white powder; ash free it weighed 12·08 g. (corrected) and contained 13·15% N. Tests for the presence of dicarboxylic acids and bases by the methods detailed more fully in the corresponding section of the egg-albumin analysis were all negative. The fraction will presumably contain all the non-nitrogenous products, except those condensed to humin, which had been formed by deamination of certain of the amino-acids during the 20% HCl hydrolysis, evidence for which is revealed in the production of NH_3 in excess of the true amide-N.

Results of analysis

If present in the hydrolysate, tyrosine will be partially precipitated during the lime-ethanol treatment, so will phenylalanine and leucine if baryta be employed instead of lime. All three of these amino-acids, especially the former, will separate readily as the hydrochloride in the presence of 20% HCl; they may, in consequence, be serious contaminants of the glutamic acid hydrochloride isolated at stages 8 and 23. In the procedure we have adopted the only likely contaminant is tyrosine hydrochloride, and as this is fairly readily soluble in absolute ethanol we always make a point of thoroughly washing our products with this solvent. The various crops of glutamic acid hydrochloride cited in Table 2 were analysed for ash and total N [Chibnall *et al.*, 1943], and their purity was controlled by confirming that the titration value in the micro-Kjeldahl was exactly one-half of that obtained when the same weight of material in aqueous solution was titrated to *p*H 7·3 (blue tint with bromothymol blue); working to fine limits, 1% monoamino-acid impurity can be detected in this way. The necessary criteria for checking the purity of Cu aspartate have been discussed in a previous paper [Bailey *et al.*, 1943]: data for the various crops obtained in the present analysis are given in Table 2.

The detailed results of the complete analysis are given in Table 3. It is to be remembered that the

Table 2. *Analytical data for glutamic and aspartic acids from edestin*

	Glutamic acid hydrochloride			Copper aspartate			
						On anhydrous material	
Crops	Wt. (corrected) g.	N %	Rotation of glutamic acid in 9% HCl $[\alpha]_D$	Wt. (corrected) g.	H_2O %	Cu %	N %
1	10·722	7·63	+30·3	10·71	29·15	32·54	7·22
2	1·994	7·65	+23·4	2·192	29·35	32·70	7·22
3	0·590	7·67		1·354	29·02	32·43	7·21
4	1·511	7·61	+ 7·3	0·910	29·36	32·28	7·11
5	0·928	7·68					
	15·745			15·166			
Theory		7·63			29·4	32·69	7·19

Table 3. *Analysis of edestin*

Stage (diagram 1)	Fraction	Wt. g.	Wt. as % of protein	N g.	N as % total protein-N
4	Mercaptide precipitates:				
	Humin	0·729	1·16	0·0759	0·61
	Cystine	0·846	1·37	0·0985	0·85
	Methionine and tyrosine	0·891	1·44	0·0796	0·74
6	Crude tyrosine	2·549	4·13	0·1973	1·71
7, 22	Glutamic acid	12·658	20·50	1·203	10·44
7, 22	Aspartic acid	7·372	11·95	0·7765	6·74
2	Ammonia	1·44	2·36	1·205	10·42
11, 23	Arginine	10·31	16·69	3·320	28·81
11, 23	Histidine	1·464	2·39	0·3948	3·47
11, 23	Lysine	1·431	2·32	0·2802	2·39
16	Monoamino-acids, crop 1 (N = 11·74 %)	5·318	8·61	0·6243	5·43
20	Monoamino-acids, crop 2 (N = 10·11 %)	2·575	4·17	0·2603	2·26
21	Monoamino-acids, crop 3 (N = 11·26 %)	7·606	12·32	0·8282	7·21
29	Monoamino-acids, crop 4 (N = 13·15 %)	12·22	19·56	1·59	13·86
18	Proline	1·996	3·23	0·2428	2·11
	Total accounted for	69·41	112·22	11·18	97·05
	Unaccounted for	3·27(?)	5·31	0·34	2·95
	Taken for analysis (61·74 g. protein)	72·68	117·7	11·52	100·0

value for proline represents that actually isolated as picrate and is not to be regarded as maximal; Osborne & Liddle [1910*b*] found 4·1 %.

The overall loss amounted to 2·95 % T.P.N., and the individual losses at the various stages are recorded in Table 4. The major loss occurs during the Cu salt treatment covering stages 14–21, and there is reason to believe that this falls almost exclusively on the more insoluble monoamino-acids, particularly methionine, and subordinate amounts of humin. The overall loss to stage 8, which occurred

Table 4. *Losses recorded at certain stages of analysis (edestin)*

Stages	Origin	Total N (corrected) g.	Total N (corrected) % total protein-N
1– 8	Overall losses to the removal of the main bulk of glutamic and aspartic acids	0·0304	0·26
11, 12	Retained by organic wash liquors	0·0128	0·11
12	Removal of inorganic reagents	0·0230	0·20
14–21	Copper salt fractionation; loss chiefly monoamino-acids	0·20	1·76
26	Unaccounted for in base solution	0·0519	0·45
21–29	Overall loss (diff.)	0·0219	0·22
1–29	Total	0·34	3·0

in large part during the working at that stage itself, must fall in part on the dicarboxylic acids, but from stage 9 onwards the concentration of these, especially at stage 25, is so low relative to the other products present that their contribution to the remaining losses must be very small indeed. In a previous paper [Bailey *et al.*, 1943] we have discussed the solubility of the Ca salts of the dicarboxylic acids in the lime-ethanol procedure, and have given our reasons for the belief that in the present edestin analysis small solubility factors can be legitimately applied at stage 22. When account has been taken of these factors our final results for glutamic acid and aspartic acid are 20·70 and 12·0 % W/P respectively (Table 14), and in view of what has been said above we believe that these are within 1–2 % of the true values. Solubility corrections for the bases are discussed in Section 5.

Reverting to the data given in Table 3, we see that 69·41 g. of hydrolysis products have been identified, and that these represent 97·05 % of the total protein-N. If we assumed for reasons given above that the loss to stage 8 represents dicarboxylic acids with an average of 10 % N, that the loss covering stages 14–21 represents monoamino-acids with an average of 9 % N, and that the other losses represent amino-acids with an average of 12 % N, then calculation shows that the gross weight of unidentified products, containing 0·34 g. N, would be 3·27 g. This value has been incorporated in Table 3 and permits the deduction that 100 g. protein have given 117·7 g. hydrolysis products. The computed value, based on the total N and the peptide-N of the protein [Chibnall, 1942], is 117·8; agreement is good, considering the assumption made with respect to the humin and unidentified products in Table 3.

3. *Analysis of egg albumin*

The edestin procedure needed slight modification on account of the larger proportion of monoamino-acids in the egg albumin hydrolysate. In the description that follows only points of difference or of unusual interest will be noted.

Details of analysis

1, 2. The sample of coagulated protein [Chibnall *et al.*, 1943] contained 0·44 % ash, and ash- and moisture-free

15·76% N. 62·02 g. ash- and moisture-free protein containing 9·78 g. N were dissolved in 600 ml. 20% HCl by heating on a water-bath for 6 hr. The mixture was then boiled under reflux for a further 24 hr. and the excess HCl removed in the usual way. Acid humin was then removed and washed 3 times with cold and 3 times with hot water; it contained 7% ash. The ash-free product weighed 0·862 g. and contained 0·0633 g. N. To avoid the withdrawal of a large aliquot from the main hydrolysate to determine alkali humin, part of which is carried down in the mercaptide precipitate, a separate small hydrolysis under identical conditions was carried out for this purpose. Alkali humin was determined by adding CaO to *p*H 7·5. The weight, ash-free, and calculated in terms of the main hydrolysate, was 1·061 g. and the total N 0·0652 g. The filtrate was deep straw-coloured, showing that all the humin had not been removed.

3, 4, 5. As 0·0162 g. of alkali humin-N was removed in the lime-ethanol treatment at stage 8 it is assumed that the remainder of that estimated above has been carried down in the mercaptide precipitate. The latter (wt. 50·5 g.) contained organic-S in excess of that due to cystine and by difference 0·3% w/P of tyrosine. It is clear, therefore, that as in the case of edestin the conditions during the precipitation have caused the separation of the less soluble monoamino-acids or their copper complexes. To ascertain whether aspartic acid or arginine were concerned, 10 g. of the product was dissolved in 250 ml. HCl, Cu^+ removed with H_2S, the precipitate of Cu_2S and humin well washed and the clarified solution reduced *in vacuo* to remove excess HCl. Treatment with flavianic acid by the method of Vickery [1940] gave 21·4 mg. recrystallized material [Lucas & Beveridge, 1940] which was definitely not arginine monoflavianate, for it did not possess the characteristic golden lustre and on attempting a second recrystallization (see Section 5) it remained in solution. The flavianate mother liquors were collected, excess flavianic acid removed as described in Section 5, and the lime-ethanol treatment for dicarboxylic acids applied from an aqueous volume of 18 ml. The Ca precipitate, when treated as in stage 8, gave a solution containing 42·2 mg. N and from this various crops of impure Cu cysteate were separated, but a careful search failed to give evidence for the presence of Cu aspartate. The final data for the composition of the mercaptide precipitate are given in Table 5. The mercaptide filtrate, still somewhat coloured, was freed from Cu^+ with H_2S without the addition of HCl, consequently the concentration of Cl^- at the 1st lime-ethanol stage was very low.

Table 5. *Composition of the organic material in the mercaptide precipitate (egg albumin)*

	Wt. g.	Wt. as % of protein	N g.	N as % total protein-N
Alkali humin	0·74	1·20	0·0490	0·50
Cystine	1·10	1·78	0·1282	1·31
Methionine	1·60	2·59	0·1582	1·62
Monoamino-acids (diff.)	0·59	0·95	0·0589	0·60
	4·03	6·52	0·3943	4·03

7, 8, 9. Two successive treatments with lime-ethanol were made from aqueous volumes of 500 ml. There was insufficient Cl^- present at the second operation to flocculate the precipitate, and it was necessary to add 8 g. crystalline $CaCl_2$. On redissolving the precipitate the residual lime retained 0·0162 g. alkali humin-N. During the treatment with HCl to effect the separation of G-HCl much darkening of the solution occurred, due presumably to the decomposition of the glucosamine moiety of the polysaccharide present in egg albumin [cf. Neuberger, 1938]. As a result 0·157 g. acid humin, containing 0·0122 g. N, was removed prior to the 3rd treatment with lime-ethanol, which was made from an aqueous volume of 500 ml. The 4th treatment was also made from a similar volume, and in the final clearing of the material recovered from the precipitate it was necessary to remove $SO_4^=$ (from the protein ash) as $CaSO_4$ with ethanol. From all these operations 4 crops of Cu-A, containing aspartic acid equivalent to 7·74% w/P, and 4 crops of G-HCl, containing glutamic acid equivalent to 13·89% w/P, were obtained. The final filtrate contained (corrected) 0·0932 g. N of which 0·0664 g. (≡0·68% T.P.N.) was amino-N. The overall loss to the end of stage 8 was 14·7 mg. N (17·9 mg. N corrected) and the flow sheets showed that this occurred in large part during the separation of the glutamic and aspartic acids.

9, 10, 11. The precipitation of the bases with P.T.A. was made from a total volume of 2 l. As the hydrolysate contained such a high proportion of monoamino-acids the base solution, freed from P.T.A., was reprecipitated from a total volume of 1450 ml., the mixture being allowed to stand at 0° overnight. It is doubtful if any benefit resulted from taking this precaution, as the residual solubility of the arginine phosphotungstate was considerable: the final base solution nevertheless was free from extraneous-N (see Section 5). The whole operation, including the removal of P.T.A. from the two filtrates, resulted in a loss of only 8·7 mg. N (11·1 mg. N corrected).

The loss on removing all inorganic reagents and excess Cl^- was 7·4 mg. N (10·1 mg. corrected). On evaporation of the clarified solution 3 crops of monoamino-acids were removed.

14–21. The filtrate from stage 13 was brownish, and clearly contained humin, part of which probably may have resulted from the operations of stage 8. On treating the solution with excess $CuCO_3$ to make the Cu salts a small reduction to Cu_2O occurred, presumably due to glucosamine or its derivatives. The extraction of the dry salts with ethanol and cold water proceeded smoothly and but little material was subsequently removed from the residue by treatment with boiling water, probably because the amino-acids concerned had been separated at stage 13. On removal of Cu^{++} from the various fractions the CuS retained 50 mg. humin-N. The separation of proline, at the picric acid stage, was more difficult than in the case of edestin on account of the presence of hydroxyproline, and fractional crystallization was necessary. The yields of the two picrates (m.p. 153° and 189°) were equivalent to 1·632 g. of proline or 2·63% w/P and 0·262 g. hydroxyproline or 0·42% w/P respectively. The overall loss in the complete Cu treatment was 0·144 g.; as in the case of edestin we believe the major part of this to be due to the insoluble Cu salt of methionine.

22, 23. The collected solutions were treated with a 1/10 vol. conc. HCl and gently boiled for 2 hr. to convert any pyrrolidone carboxylic acid present to glutamic acid. Excess HCl was then removed and the lime-ethanol treatment applied 3 times in succession from 200 ml. of aqueous solution. The clarified solution from the ultimate precipitate contained 0·2057 g. N (0·385 g. corrected) of which only 0·147 g. was amino-N, indicating the presence of much

arginine due to recrystallization of the base precipitate at stage 10. G-HCl crop 5, and a fraction of Cu-A were separated: the latter needed recrystallization. The lime-ethanol treatment was then applied once more from 200 ml.; the clarified solution from the precipitate contained 0·0807 g. N and 0·0597 g. amino-N. G-HCl crop 6, and a fraction of Cu-A were separated; the latter was added to that mentioned above to give Cu-A crop 5. The final mother liquor contained 58 mg. of amino-N (corrected) equivalent to 0·59% T.P.N. Much of this was probably due to serine, as egg albumin contains many per cent of this hydroxy-amino-acid [Martin & Synge, 1941]. The loss during these operations was 18·7 mg. (corrected), part of which must fall on the dicarboxylic acids.

24, 25, 26. The base precipitation was made from a total volume of 290 ml. The precipitate was filtered off, washed on the filter paper and then redissolved (incompletely) by warming with 180 ml. of P.T.A. wash solution for 10 min. at 80°. The mixture was allowed to stand at room temperature for 2 hr. and then filtered off. The filtrate was shown later to contain much arginine, and as in stage 10, it is doubtful if this recrystallization was of any advantage.

27, 28, 29. The clarified solution on evaporation to a small volume gave M.A. crop 5, containing 12·31% N and on taking the mother liquor to dryness in a vacuum desiccator there was obtained a final fraction containing 3·7% ash. Ash-free, this residual product weighed 4·34 g. (9·297 g. corrected).

As the base precipitate at stage 25 had been recrystallized we suspected that a small amount of arginine phosphotungstate had remained in solution. Accordingly 2·604 g. of the final residue was dissolved in 75 ml. water and arginine flavianate precipitated by the method of Vickery [1940], using 2·4 g. flavianic acid. The yield of arginine flavianate (tested for purity as described in Section 5) was equivalent to 0·1358 g. arginine (corrected). The excess flavianic acid was removed from the filtrate by strongly acidifying with HCl and extracting with amyl alcohol-ether mixture. The aqueous solution freed from excess HCl was then submitted to the lime-ethanol treatment from a volume of 50 ml. The clarified solution from the precipitate contained 38·7 mg. N, equivalent to 0·88% T.P.N. and it was considered that the major part of this would be due to serine. Abderhalden & Kautzsch [1912] observed that aspartic and glutamic acids were precipitated by Hg acetate, and we have found that this is so even in the presence of 10 times the equivalent of serine. The above-mentioned solution was treated accordingly and the precipitate freed from Hg^{++} with H_2S. The clarified solution (8·9 mg. N≡0·3% T.P.N.) gave a small amount of good crystallin Cu aspartate, in amount (corrected) equivalent to only 17 mg. of aspartic acid or 0·03% W/P and in excellent agreement with the calculated solubility correction [Bailey *et al.*, 1943] for stage 23. No glutamic acid hydrochloride could be isolated.

The mother liquors and the various filtrates were then collected, all reagents removed, and the clarified solution submitted to electrodialysis by Mr A. H. Gordon. Only 3 mg. N (10·6 mg. N corrected≡0·11% T.P.N.) passed into the cathode compartment, showing that no appreciable basic-N was present. The object of this last experiment was to demonstrate the absence of ornithine, for the properties of this base are such that it would almost certainly have escaped precipitation with phosphotungstic acid at stages 10 and 25. In Table 7 monoamino-acids crop 7 represents the residual 9·297 g. less the amount of arginine and aspartic acid subsequently isolated.

Results of analysis

The analytical data for the various crops of glutamic acid hydrochloride and copper aspartate are given in Table 6. Calculation shows that the glutamic acid hydrochloride isolated at stage 8 (i.e. crops 1–4) represents 8·269 g. of *l*(+)-glutamic acid and 0·384 g. of *dl*-glutamic acid; the corresponding weights for stage 23 are 0·721 and 0·6 g. respectively, showing that the solubilities of the two Ca salts in the modified Foreman procedure are similar [Bailey *et al.*, 1943] and refuting the contrary claim of Kögl, Erzleben & Akkermann [1939]. 9·8% of the total glutamic acid isolated at stages 8 and 23 can be regarded as fully racemized.

The detailed results of the analysis are given in Table 7. The main working losses have been indicated in the text and if these are evaluated as in the case of edestin it is possible to suggest the data given in the table for the products unaccounted for. The weight of hydrolysis products given by 100 g. protein is 117·8 g., a value that cannot be accurate to ±0·5 g. on account of the difficulty of assessing the alkali humin fractions, into which, with the acid humin, most of the mannose and glucosamine of the polysaccharide moiety must have passed through condensation with tryptophan and subordinate amounts of cystine and tyrosine [Lugg; see Chibnall, 1939, p. 162]. The computed value is 117·2 g. [Chibnall, 1942]. When account is taken of the small solubility corrections applicable at stage 22

Table 6. *Analytical data for glutamic and aspartic acids from egg albumin*

	Glutamic acid hydrochloride			Copper aspartate			
						On anhydrous material	
Crops	Wt. (corrected) g.	N %	Rotation of glutamic acid in 9% HCl $[\alpha]_D$	Wt. (corrected) g.	H_2O %	Cu %	N %
1	7·425	7·65	+31·7	6·319	29·14	32·5	7·15
2	1·762	7·65	+31·3	2·272	29·32	32·5	7·20
3	0·735	7·64	+29·7	0·845	29·3	32·5	7·19
4	0·770	7·61	+13·6	0·479	28·6	32·6	7·15
5	0·884	7·67	+26·1	0·459	29·2	32·4	7·18
6	0·727	7·66	+ 5·5	—	—	—	—

Table 7. *Analysis of egg albumin*

Stage (diagram 1)	Fraction	Wt. g.	Wt. as % of protein	N g.	N as % total protein-N
2, 8	Acid humin	1·019	1·64	0·0755	0·77
3, 8, 20, 21	Alkali humin	2·110	3·40	0·1344	1·37
4	Mercaptide precipitates:				
	Cystine	1·100	1·77	0·1282	1·31
	Other amino-acids	2·190	3·54	0·2171	2·22
6	Crude tyrosine	1·511	2·44	0·1184	1·21
2, 8, 26	Ammonia	1·071	1·73	0·8909	9·11
7, 22	Glutamic acid	9·913	16·00	0·9428	9·64
7, 22	Aspartic acid	5·019	8·10	0·5294	5·41
11, 23, 29	Arginine	3·483	5·62	1·1211	11·46
11, 23	Histidine	0·901	1·45	0·2443	2·50
11, 23	Lysine	3·136	5·06	0·6010	6·15
13	Monoamino-acids, crop 1 (N = 10·71 %)	10·120	16·32	1·0830	11·08
13	Monoamino-acids, crop 2 (N = 11·04 %)	2·557	4·12	0·2822	2·89
13	Monoamino-acids, crop 3 (N = 11·35 %)	5·443	8·78	0·6179	6·32
16	Monoamino-acids, crop 4 (N = 10·88 %)	3·466	5·59	0·3772	3·86
16	Monoamino-acids, crop 5 (N = 10·85 %)	2·589	4·18	0·2826	2·89
28	Monoamino-acids, crop 6 (N = 12·31 %)	3·624	5·84	0·4650	4·76
28	Monoamino-acids, crop 7 (N = 12·25 %)	9·142	14·74	1·1381	11·64
18	Proline	1·632	2·63	0·1920	1·96
19	Hydroxyproline	0·262	0·42	0·0383	0·40
1–29	Total accounted for	70·29	113·35	9·50	96·95
	Unaccounted for	2·55(?)	4·13	0·28	2·86
	Taken for analysis (62·02 g. protein)	72·84	117·5	9·78	100·0

the final results for glutamic acid and aspartic acid are 16·1 and 8·13 % w/P respectively (Table 14). As the gross losses at stages 8 and 23 totalled only 36·6 mg. N we believe that these results are within 1–2 % of the true values.

Throughout the analysis we have kept in mind Fearon's [1939] recent claim that egg albumin contains citrulline. The unhydrolysed protein gave the bright purple-red colour with diacetyl monoxime, as he states. After acid hydrolysis the colour was redder, yet it did not match the characteristic red given by citrulline itself; the colour moreover faded rapidly, whereas that given by citrulline is stable for many days. This last difference became more pronounced as the hydrolysate was fractionated, and the various crops of monoamino-acids described in the text and in Table 7 failed to give the test at all. Citrulline is not very stable on acid hydrolysis, as shown by the fact that a sample of 0·2 g., when boiled with 10 ml. of 20 % HCl for 22 hr., yielded 14·2 % of the total N as amide-N; even so we should have expected to find evidence for it in the amino-acid mixtures recovered from the Cu salts at stages 16, 20 or 21 if any appreciable amount had been present in the hydrolysate. Our evidence therefore does not support Fearon's claim.

4. *Analysis of β-lactoglobulin*

In light of the experience gained with the other two proteins the procedure used in this case was considerably simplified.

Details of analysis

1, 2. 30·61 g. moisture- and ash-free coagulated protein, containing 4·76 g. N or 15·55 %, were heated on the water-bath with 250 ml. 20 % HCl until solution was effected and then boiled under reflux, for an inclusive period of 24 hr.

4. The mercaptide precipitated was analysed for N but not for S or for humin.

7, 8, 9. Three lime-ethanol treatments were made in succession from an aqueous volume of 250 ml. before the separation of the dicarboxylic acid derivatives was attempted. The clarified precipitate contained 1·042 g. N (corrected) ≡ 21·9 % T.P.N. Repeat precipitations were made in due course from 200 and 100 ml. respectively.

9, 10, 11. The base precipitation was made from a total volume of 1600 ml. The total N in the precipitate was 0·9282 g. (corrected) (see Section 5).

13. Four crops of monoamino-acids were obtained, weighing 4·062, 1·360, 2·951 and 1·557 g. and containing 10·07, 10·67, 11·02 and 11·81 % N respectively.

14–21. The Cu salt procedure was omitted, as a determination of amino-N on the filtrate from 13 indicated that the proline and hydroxyproline content must be fairly small.

22, 23, 24. The lime-ethanol treatments were made in succession from aqueous volumes of 100, 200 and 200 ml. respectively before the separation of the dicarboxylic acid derivatives was attempted. The clarified precipitate contained 0·0708 g. N ≡ 1·49 % T.P.N. A final treatment from 50 ml. was made in due course. The ultimate mother liquor contained 13·3 mg. N (corrected), equivalent to only 0·28 % T.P.N.

25, 26. The 2nd base precipitation was made from a total volume of 265 ml. The total N in the precipitate was 54·2 mg. all of which was accounted for as base (see Section 5).

28, 29. Two crops of amino-acids and a final residue were obtained; these weighed 1·745, 3·59 and (ash not deducted) 5·94 g. and contained 11·37, 12·38 and 10·5 % N respectively. The latter product was treated with flavianic acid, but no arginine salt could be separated.

Results of analysis

The data given in Table 8 summarize the fractionation of the N; as the mercaptide precipitate was not examined in detail it is not possible to summarize the hydrolysis products as in the case of the other two proteins. The overall loss was 59·9 mg. N (corrected), while the losses on working stages 8 and 23 were 5·0 and 1·6 mg. N respectively, jointly equivalent to 0·14 % T.P.N., part of which must fall on the dicarboxylic acids. The final values for arginine, histidine, lysine, glutamic acid and aspartic acid are 2·89, 1·54, 9·75, 21·32 and 9·83 % w/p respectively. If the small solubility corrections for stage 22 be applied [Bailey *et al.*, 1943] the two latter values are raised to 21·51 and 9·88 % respectively (Table 14) and we believe these are accurate to 1–2 %. The solubility corrections for the bases are discussed in Section 5.

Table 8. *Analysis of β-lactoglobulin*

Stage (diagram 1)	Fraction		N g.	N as % total protein-N
2	Ammonia		0·3593	7·55
3	Mercaptide precipitate		0·2090	4·39
8	Glutamic acid	0·5982 g.	0·6199	13·03
23	Glutamic acid	0·0217 g.		
8	Aspartic acid	0·2912 g.	0·3159	6·65
23	Aspartic acid	0·0247 g.		
8	Crude tyrosine		0·0220	0·46
11	Arginine	0·2623 g.	0·2799	5·88
26	Arginine	0·0176 g.		
11	Histidine	0·1076 g.	0·1247	2·62
26	Histidine	0·0171 g.		
11	Lysine	0·5537 g.	0·5719	12·01
26	Lysine	0·0182 g.		
13	Monoamino-acids in 4 crops		1·0637	22·35
28, 29	Monoamino-acids in 3 crops		1·1332	23·81
	Total accounted for		4·6995	98·75
1, 29	Unaccounted for		0·0599	1·25
	Taken for analysis		4·76	100·0

5. Analysis of the base fractions

The present study is to be regarded as a continuation of that of Tristram [1939] on the reliability of various methods for estimating the basic amino-acids in protein hydrolysates. Tristram favoured the use, with certain minor modifications, of Block's [1938] small-scale adaptation of the modified Kössel procedure devised by Vickery and his co-workers [Vickery & Leavenworth, 1928; Vickery & Shore, 1932; and Vickery & White, 1933] and he tested this, *inter alia*, against appropriate mixtures of the bases representing 2–3 g. of protein. The losses at the various stages in the analysis were determined. Having allowed for the solubility of arginine silver [1·1 mg. N/100 ml., Gulewitsch, 1899], the overall loss in the estimation of arginine was 2·6 mg. N, due in part to precipitation with the histidine silver at *p*H 7·2 and in part to incomplete precipitation as arginine silver at *p*H 12–13. The overall loss in the estimation of histidine was small (0·3 mg. N/100 ml.) while that for lysine (1·6 mg. N/100 ml.) was but little more than that due to the solubility of lysine phosphotungstate. Tristram considered that corrections to allow for these overall losses could be legitimately applied when analysing a mixture of the three bases, but that in the case of protein hydrolysates the validity of such corrections must remain a matter of conjecture until new methods of analysis were found which would permit a direct determination of the solubility factors concerned. Examination of the base solutions obtained during the fractionation of the hydrolysates described in Sections 2–4 has enabled us to investigate these solubility factors in detail.

Base fraction of β-lactoglobulin

The two base fractions described in Section 4 were analysed by the Tristram-Block procedure mentioned above, the only modifications being the employment of Block's [1940] nitranilic acid method for the estimation of histidine and the thorough washing of all inorganic precipitates as described in Section 2. The purity of the arginine flavianate, which might be contaminated with the histidine salt, was controlled by recrystallization under standard conditions detailed later in our discussion of Vickery's method for arginine. Arginine nitranilate is fairly soluble under Block's conditions, but the lysine salt is as insoluble as that of histidine itself. As nitranilic acid is readily decomposed in hot water [Town, 1936] the purity of the isolated histidine salt was controlled in the following way. 0·1 g. material and 25 ml. water were placed in a 100 ml. beaker, which was then partly immersed for exactly 4 min. in a water-bath at 65°. Solution of the salt is readily effected on stirring, and if there be an insoluble residue the presence of Ba nitranilate may be suspected. The beaker was then removed from the bath, 25 ml. of methanol were run in with stirring, and crystallization allowed to proceed for 2 days at 2°. The filtered, washed and dried product should weigh 0·0980 g. Under the conditions of a base analysis it is improbable that lysine would pass into the histidine fraction, but the procedure serves to detect the presence of the Ba salt, a possible contaminant.

Analysis of 1st base solution. The aliquot used contained 0·3664 g. N; as this amount of basic-N was approximately 3 times that present in the hydrolysate of 2–3 g. protein the separation into the 3 base fractions was made from 3 times the respective volumes employed by Tristram. Excess HCl was removed by evaporation *in vacuo*, and the solution freed from Cl^- with Ag_2O and H_2SO_4 at *p*H 3·0

in the usual way. The arginine, histidine and lysine fractions contained 0·1034, 0·0394 and 0·2207 g. of N respectively. The N recovered in the form of base derivatives in this first analysis is given in Table 9, and allowing solubility corrections (300 ml.) for lysine phosphotungstate and arginine silver the N unaccounted for was 9·5 mg. The mother liquors of the 3 derivatives were collected, reduced *in vacuo* to a few ml., strongly acidified with HCl and the 3 acidic reagents extracted by the amyl alcohol-ether procedure for the removal of excess phosphotungstic acid, an operation that can be conducted without loss of basic-N. The clarified solution, freed from HCl and Cl^- and containing 7·0 mg. N, was then passed once again through the Tristram-Block procedure, using a third of Tristram's volumes. The weight which may not be true as the amino-acid composition and concentration in the respective solutions was not the same, the amounts of the bases isolated from the 2nd base solution (column 1) represent the solubilities in 1335 ml. From these the solubility corrections for the 2nd and 1st precipitations (columns 2 and 3) have been calculated. The original Van Slyke corrections for the latter are given in column 4. Comparison is not strictly valid as we did not use P.T.A. purified by Winterstein's method, but our low value for histidine is what would be expected from the more recent work of Van Slyke *et al.* [1942]. The two arginine values show fair agreement, while the great difference between those for lysine suggests that the amino-acid mixture has influenced the solubility in our case. The final values for

Table 9. *Analysis of the base fractions of β-lactoglobulin*

	1st base solution		2nd base solution		
	N g.	N (corrected) as % total protein-N	N g.	N (corrected) as % total protein-N	Total base N as % total protein-N
Aliquot taken for analysis	0·3664	19·50	0·0496	1·10	20·60
Arginine isolated as flavianate, 1st analysis	0·0975	—	0·0135	—	(5·43)
Correction for solubility of arginine silver, 1st analysis	0·0033	—	0·0011	—	—
Correction for overall losses, 1st analysis	0·0026	—	0·0026	—	—
Total arginine	0·1034	5·51	0·0172	0·37	5·88
Histidine isolated as nitranilate, 1st analysis	0·0375	—	0·0162	—	—
Histidine isolated as nitranilate, 2nd analysis	0·0048	—	—	—	—
Total histidine	0·0423	2·26	0·0162	0·36	2·62
Lysine isolated as picrate, 1st analysis	0·2170	(11·53)	0·0159	—	(11·88)
Correction for solubility of lysine phosphotungstate	0·0015	—	0·0008	—	—
Total lysine	0·2185	11·63	0·0166	0·37	12·0
Total base accounted for	0·3642	19·38	0·50	1·11	20·49

of histidine nitranilate obtained in the 2nd analysis was 40·8 or 43·8 mg. corrected to the 1st base solution, equivalent to 4·8 mg. histidine-N. This amount of histidine must have passed into the arginine fraction in the 1st analysis. No flavianate was obtained from the arginine fraction of the 2nd analysis. The complete results for the 1st base solution are set out in Table 9. As it is clear that no appreciable amount of extraneous non-basic material is present we have included Tristram's correction of 2·6 mg. for overall losses of arginine-N, though we worked to 3 times his volumes; such an amount is, in fact, insufficient to lead to any recovery of arginine flavianate in the 2nd analysis.

The 2nd base solution was passed once through the Tristram-Block procedure, working to Tristram's volumes. Again allowing a correction of 2·6 mg. for overall losses of arginine-N the recovery of basic-N is complete, as shown in Table 9. It may be mentioned that in all the above-mentioned analyses the arginine flavianate and histidine nitranilate were shown to be pure by the solubility test mentioned earlier, and the lysine picrate, without recrystallization, exploded sharply at 266–267°. The overall correction for working losses applied to arginine accounts for 0·2% T.P.N. and this must be considered the one uncertainty in the full analysis.

It is of interest to consider the solubility of the phosphotungstates on precipitation of the bases. Relevant data are collected in Table 10. The volume employed in the 1st precipitation was 1600 ml. and in the 2nd 265 ml. On the assumption that the phosphotungstate solubilities in the two cases were proportional to the volumes employed, the bases given in Table 14 include the solubility corrections (Table 10, column 2) for the 2nd precipitation. We believe that these will be found to be within 2% of the true values.

Table 10. *Base phosphotungstate solubility corrections for β-lactoglobulin*

(Values quoted are in percentages of total protein-N.)

	Isolated from 2nd base solution	Solubility corrections		Van Slyke solubility corrections
		2nd base precipitation	1st base precipitation	1st base precipitation
	(1)	(2)	(3)	(4)
Arginine	0·37	0·07	0·44	0·51
Histidine	0·36	0·07	0·43	0·64
Lysine	0·37	0·07	0·44	0·08

Direct Tristram-Block analysis. 3·803 g. moisture- and ash-free protein, containing 0·5895 g. N, were hydrolysed with H_2SO_4 for 24 hr. The analysis followed the lines of those mentioned above, including the thorough washing of all inorganic precipitates. The arginine flavianate and histidine nitranilate were of satisfactory purity; the lysine picrate was recrystallized from 15 ml. water and corrected for solubility (3·4 mg. N/ml. at 0°); it exploded at 266–267°.

The collective results given in Table 11 bring out some interesting comparisons with Table 9. In the Tristram-

Table 11. *Tristram-Block analysis of β-lactoglobulin*

	Total N g.	N as % total protein-N
Arginine fraction	0·0394	
Arginine isolated as flavianate	0·0316	(5·36)
Correction for arginine silver (150 ml.)	0·0016	
Correction for overall losses	0·0026	
Total arginine	0·0358	6·07
Histidine fraction	0·1292	
Histidine isolated as nitranilate	0·0133	2·26
Lysine fraction	0·1186	
Lysine isolated as picrate	0·0681	(11·55)
Correction for overall losses	0·0016	
Total lysine	0·0697	11·82

Block analysis the arginine flavianate isolated was equivalent to 5·36% of the total protein-N, while our comprehensive procedure gave 5·43%; yet the corrected values were 6·07 and 5·95% respectively, suggesting that in the direct analysis the amount of N allowed for overall losses was a little too high. The histidine value in the Tristram-Block analysis is low and as according to our experience the total N in the histidine fraction varies enormously from protein to protein it is difficult to suggest any general overall corrections. With a mixture of bases Tristram's losses by his procedure were negligible (0·3 mg. N) and we confirm Block's finding that under the conditions that he applies nitranilic acid the precipitation of histidine in the absence of extraneous material is quantitative. Whether the low yield in the present case is to be ascribed to the increased solubility of histidine silver in the presence of the protein hydrolysis products or to that of histidine nitranilate in the presence of nearly 10 equivalents of extraneous N cannot be stated with certainty; both factors are probably operative, each to a degree that will vary from protein to protein. In the Tristram-Block analysis the lysine value based on the isolated picrate was 11·55%, in excellent agreement with the 11·53% obtained from the first phosphotungstate precipitate in the comprehensive procedure. The corrected value is a little low, as one would expect from our demonstration that the solubility of the phosphotungstate is enhanced by the presence of the amino-acids of the hydrolysate: even so the presence of much extraneous material in the lysine fraction has probably prevented the quantitative separation of the lysine as picrate in one operation [Vickery & Leavenworth, 1928].

Base fractions of edestin and egg albumin

The procedure used was the same as that described for lactoglobulin, and the results are given in Table 12. In the case of edestin the volume ratio in the two base precipitations was 9 to 1, so that the calculated corrections for the phosphotungstate solubilities in the 2nd were 0·04, 0·03 and 0·05% T.P.N. respectively. The final values for the bases are given in Table 14.

Conditions were different in the case of egg albumin, for both phosphotungstate precipitates were recrystallized. From the results given in Table 12 it would appear that on recrystallization of the 1st precipitate the arginine phosphotungstate separated as a gelatinous rather than a crystalline precipitate [Van Slyke *et al.*, 1942] and its solubility has been much enhanced. As the 2nd base precipitate was also recrystallized we inspected a similar occurrence and accordingly estimated the amount of arginine in the amino-acid fraction at stage 29 by the Vickery flavianic acid method discussed later. This fraction contained 12·1% of the T.P.N. and from it was isolated arginine equivalent to 0·41% of the T.P.N. After removal of the excess flavianic acid, and also of the residual dicarboxylic acids, from the arginine flavianate filtrate, the clarified solution gave on electrodialysis 0·11% T.P.N. in the cathode compartment. This value sets a definite limit to the residual solubilities of the 3 bases, as it will include any monoamino-acid-N which had migrated by simple diffusion. Our final results for egg albumin are therefore those given in Table 12, and are believed to be not more than 2% low.

Direct arginine analysis by the method of Vickery

In the comprehensive analyses mentioned above our original plan had been to effect a preliminary removal of arginine as flavianate by Vickery's [1940] new method so that the histidine and lysine fractions in the subsequent Tristram-Block procedure would be free from this base. The expectation was not realized, for in the absence of all the non-basic products of hydrolysis the recrystallized product was found to be heavily contaminated with the flavianates of the other two bases, and repeated recrystallization was necessary to obtain pure arginine flavianate. As this latter substance has an appreciable solubility under the conditions employed small amounts of it passed into the various mother liquors and washings, so that the incorporation of this additional step in the analysis of the base solutions defeated its own end. These findings prompted us to investigate the purity of the arginine flavianate obtained when the simple Vickery procedure for this base is applied directly to solutions containing known amounts of the three bases, and to protein hydrolysates.

Table 12. *Analysis of the base fractions of edestin and egg albumin*

	Edestin				Egg albumin			
	N as % of total protein-N				N as % of total protein-N			
	1st base solution	2nd base solution	Total	Wt. (% of protein)	1st base solution	2nd base solution	Total	Wt. (% of protein)
Arginine	28·47	0·33	28·80	16·69	8·20	2·85	11·46*	5·63
Histidine	3·20	0·27	3·47	2·39	2·27	0·23	2·50	1·45
Lysine	2·01	0·38	2·39	2·32	5·98	0·17	6·15	5·06

* Included 0·41% isolated as flavianate at a later stage than the 2nd base precipitation.

Precipitation of arginine as flavianate. 30 ml. of a solution of arginine dihydrochloride containing 0·2084 g. arginine were treated with 1·84 g. (5 mol.) flavianic acid according to Vickery's directions. After 4 days at 2° the precipitate was filtered off, washed with 30 ml. of water saturated at room temperature with arginine monoflavianate and recrystallized from 25 ml. via NH_3 and H_2SO_4. The precipitate weighed 0·5805 g. (theory 0·5845 g.) and on further recrystallization 5 times successively under the same conditions, the losses in weight were 3·0, 3·7, 2·2, 3·2 and 2·4 mg. respectively, or an overall mean of 3·0 mg. If this correction be applied to the weight of flavianate obtained after 1 recrystallization, giving a total of 0·5840 g. in all, the recovery is almost theoretical and confirms Vickery's claim that the diflavianate, in which form most of the arginine separates in the initial stage, is insoluble in the presence of an excess of flavianic acid. A similar experiment, using a volume of 50 ml. for the preliminary separation and for all subsequent recrystallizations, gave a mean overall loss of 4·0 mg.

Precipitation of arginine as flavianate from a mixture of bases. (1) The solution (30 ml.) contained 0·2084 g. arginine, 0·171 g. lysine and 0·0505 g. histidine, the relative proportions being approximately the same as in egg albumin. It was treated with 1·84 g. flavianic acid and the recrystallized product (from 25 ml.), which appeared to consist of plates with the golden yellow lustre characteristic of arginine monoflavianate, weighed 0·6465 g. The losses on successive recrystallization were 46·9, 15·8, 6·8, 7·9, 3·1, 2·2 and 3·1 mg. suggesting that 5 recrystallizations in all were necessary to give pure arginine monoflavianate. Applying the overall correction (8 × 3·0 mg.) the corrected weight was 0·5848 g. giving a theoretical estimation of the arginine present. (2) The solution (30 ml.) contained the same weight of arginine as before, and the other two bases in the relative proportions that occur in edestin. After 1 recrystallization the weight of the product was 0·595 g. and the losses in 2 subsequent recrystallizations were 19·2 and 3·0 mg. respectively. Applying the overall correction of 9·0 mg. the yield of pure monoflavianate was 0·5818 g. In both these experiments the arginine content, calculated from the product obtained on 1 recrystallization, was in excess of that known to be present, whereas if the recrystallization be continued until the operational loss is no more than 3·0 mg. and the appropriate correction be applied, the recovery is satisfactory. These results are not strictly in keeping with Vickery's deductions, and it became of interest to apply a similar treatment to products obtained directly from protein hydrolysates. In the following experiments Vickery's directions were adhered to as closely as possible and the flavianate was separated by using 5 mol. flavianic acid/mol. arginine present.

Arginine content of edestin. Details of four experiments, using different concentrations of protein, are given in Table 13. In the 1st the arginine content of the protein, calculated from the weight of recrystallized flavianate, was 16·75%, a value in excellent agreement with that of Vickery [1940] and also with that obtained in our comprehensive analysis (Table 3). The product, nevertheless, was not pure arginine monoflavianate, and in each of the four replicate determinations made it was necessary to recrystallize 5 times in all before the operational loss in weight was reduced to 4 mg. Allowing for overall losses (5 × 4 = 20 mg.) the corrected arginine content of the protein was 16·23 ± 0·03%. The other three experiments, in which different concentrations of protein were used, gave corrected values in close agreement with this figure.

This result was somewhat disconcerting, for our own experiments with a solution of arginine, confirming those of Vickery, show that the product obtained after only 1 recrystallization does not contain any arginine diflavianate. Appropriate Tristram-Block analysis of the collected edestin mother liquors from the 2nd to the 5th recrystallization moreover failed to disclose the presence of any appreciable amount of histidine. The extraneous material was clearly some other amino-acid flavianate, and indirect evidence in support of this is furnished by the fact that in these edestin experiments, and also in those of other proteins quoted later, the 1st recrystallization product was slightly hygroscopic (as Vickery records). According to our experience pure arginine monoflavianate, after drying in the oven at 102° and cooling to room temperature in a desiccator, gains no more than 1 mg./0·5 g. when exposed for 3 days to the atmosphere of the laboratory. In a recent paper, Vickery, Smith, Hubbell & Nolan [1941] cite experiments with water-melon globulin in which low results (17·44% as against 17·90%) were obtained when the original hydrolysate-flavianic acid mixture, after standing at 6–7° for 4 days, was allowed to stand at room temperature for a short time before filtration. They attributed this to resolution of arginine diflavianate, but from our experience we would suggest that it was the contaminants mentioned above that had been redissolved and that the lower value of 17·44% is the more reliable. In the experiments quoted in Table 13 the hydrolysate-flavianic acid mixture was allowed to stand for 4 days at +2° before filtration; a slightly higher temperature (6–7°) did not significantly affect the results.

Arginine content of egg albumin. The value based on the once recrystallized product is in close agreement with that obtained by Vickery [1940] if allowance is made for the N contents of the proteins concerned. The corrected value, based on the 4 times recrystallized product, is in agreement with that obtained during our comprehensive analysis (see Table 12). For this protein the differences concerned are small.

Arginine contents of horse and cattle haemoglobin. Clarification with charcoal of these two protein hydrolysates did not remove all the haem, which separated at once on adding flavianic acid to the aliquot (25 ml.) used for analysis. The precipitate was centrifuged off immediately and well washed. The collected centrifugate and washings (45 ml.), on standing, gave a voluminous precipitate of yellow crystalline material containing large amounts of histidine and lysine flavianates. After filtering off, the product was liberally washed with the usual wash solution, but in spite of this the recrystallized material was still heavily contaminated and 4 additional recrystallizations were needed to give pure arginine monoflavianate. The corrected value (Table 13) for the arginine content of horse carboxyhaemoglobin is a little higher than the 3·59 ± 0·07% recorded by Vickery [1940], while that for cattle haemoglobin is much higher than the 3·1% (on the globin) recorded by Bergmann & Niemann [1937].

Arginine content of amandin. Accepting 18·75% as the total N content of this protein [Chibnall *et al.*, 1943] our corrected value for arginine is 12·54 ± 0·05%. The value for arginine-N is 21·63% T.P.N.; Vickery [1940] records 22·1% T.P.N.

All these findings lead us to the conclusion that the Vickery procedure, if modified as we suggest, will give a high yield of arginine monoflavianate of unquestionable

Table 13. *Results of arginine analyses by Vickery's method*

Protein used	Edestin				Egg albumin	Horse carboxy-haemoglobin	Cattle haemoglobin
Wt. of protein in aliquot of hydrolysate (g.)	2·935	0·8288	0·97	0·97	2·665	3·565	3·76
Vol. used for precipitation (ml.)	50	50	25	50	25	45	45
Conc. of protein (g./100 ml.)	5·86	1·66	3·88	1·94	10·66	7·92	8·35
No. of determinations	4	2	4	2	4	2	3
Vol. used for recrystallization of flavianate (ml.)	50	50	25	50	25	50	50
Wt. of recrystallized flavianate (g.)*	1·379	0·381	0·5	0·445	0·4264	{0·4593 {0·4895	0·4516
Arginine content of protein (%)	16·75	16·4	16·5	16·34	5·71	—	—
No. of additional recrystallizations of flavianate	4	2	3	2	3	4	3
Final wt. of flavianate (g.)†	1·316	0·366	0·431	0·428	0·4105	0·3571	0·3659
Overall correction (g.)	0·02	0·012	0·012	0·012	0·012	0·02	0·016
Corrected arginine content of protein (%)	16·23	16·31	16·31	16·20	5·65	3·75	3·62

* In each analysis (except those of the haemoglobins) the replicates did not differ by more than 10 mg.
† In each analysis the replicates did not differ by more than 1 mg.

purity, yet we are not convinced that in all cases, especially when the hydrolysate contains a large amount of arginine, the separation is strictly quantitative. In other words, the method may not indicate with absolute certainty the number of arginyl residues in the protein molecule.

DISCUSSION

It will be seen from Tables 3, 4, 7 and 8, and from the text, that the working losses in the procedure we have adopted for the separation and estimation of the dicarboxylic acids and bases in protein hydrolysates have been reduced to what are probably the lowest possible limits. In the analysis of β-lactoglobulin, which required about 900 working hr. to take to completion, the gross overall loss of N amounted to only 1·25% T.P.N., a remarkable achievement considering the number of operations concerned, for it is less than that which most previous workers have incurred during the preliminary removal of the mineral acid used for hydrolysis. With edestin and egg albumin the corresponding stepwise losses were no greater, but the total overall loss was enhanced by the application of the Cu salt procedure for the removal of certain of the mono-amino-acids and proline. Later experience suggests that this loss was greater than it need have been, for we used an unnecessarily large amount of $CuCO_3$ to make the Cu salts with the result that the relatively small amount of N retained by the bulky residue was not readily characterized. At the time these operations were performed the almost complete insolubility of the Cu salt of methionine in boiling water was not realized. In any future analysis we would suggest that the Cu salts at stage 14 be made by the cautious addition of $CuCO_3$ to the boiling solution under conditions that would permit of rapid filtration of the excess before the separation of this very insoluble salt of methionine, which could thus be obtained free from extraneous material. If the protein contains a polysaccharide moiety, as in egg albumin, or polyuronide impurities as in our forage crop preparations, reduction of Cu^{++} to give Cu_2O will occur almost immediately, but it should be possible to remove this with the slight excess of $CuCO_3$ by filtration.

The complete procedure is admittedly very laborious, yet we feel that our final data (Table 14) for edestin, egg albumin and β-lactoglobulin will be of value to those interested in protein analysis, for the errors involved are known within small limits and the results can thus be used to assess the accuracy of new or existing methods of estimation which are less onerous to perform. We have, indeed, already attempted this in our investigations on the dicarboxylic acids [Bailey *et al.*, 1943] and the bases (Section 5).

Table 14. *Dicarboxylic and basic amino-acid analysis of edestin, egg albumin and β-lactoglobulin*

(Small residual solubility corrections have been applied.)

	Edestin		Egg albumin		β-Lactoglobulin	
	N as % total protein-N	Wt. as % of protein	N as % total protein-N	Wt. as % of protein	N as % total protein-N	Wt. as % of protein
Glutamic acid	10·54	20·70	9·70	16·10	13·14	21·51
Aspartic acid	6·77	12·0	5·43	8·13	6·68	9·88
Arginine	28·85	16·71	11·44	5·63	5·95	2·89
Histidine	3·50	2·41	2·50	1·45	2·69	1·54
Lysine	2·44	2·37	6·15	5·06	12·07	9·75

SUMMARY

1. A method for the estimation of the dicarboxylic and basic amino-acids in protein hydrolysates is described, in which these amino-acids are isolated as pure derivatives and in quantitative yield by step-wise removal without overall loss.

2. The cardinal point in the procedure is that no reagent may be introduced into the main hydrolysate unless it can be quantitatively removed at a later stage without appreciable loss of N. Cu^{+} and Cu^{++} may be introduced into the hydrolysate and subsequently removed as Cu_2S and CuS respectively at any stage in the analysis; $SO_4^{=}$ must not be removed as $BaSO_4$, or Cl^{-} as AgCl until the hydrolysate has been cleared of cystine and the main part of the dicarboxylic acids.

3. In the analytical procedure adopted the cystine is first removed as the cuprous mercaptide of cysteine. The major part of the dicarboxylic acids is then isolated by a modification of Foreman's lime-ethanol method. Following this the bases are removed by precipitation with phosphotungstic acid and then estimated by the gravimetric procedure of Tristram-Block. The solubility factors inherent in the Foreman treatment for the dicarboxylic acid and in the phosphotungstate precipitation of the bases are overcome by repeating these operations at a later stage when the major part of the monoamino-acids and proline has been removed by various means from the collected mother liquors.

4. The method has been applied in turn, with modification suggested by experience, to hydrolysates of edestin, egg albumin and β-lactoglobulin; the overall losses of N were 2·95, 2·86 and 1·25% of the total protein-N respectively. The final values for glutamic acid, aspartic acid, arginine, histidine and lysine are given in Table 14.

5. The accuracy of the Tristram-Block procedure for the direct estimation of the bases and of Vickery's flavianic acid method for the direct estimation of arginine are criticized in light of experience.

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The Tryptophanase-tryptophan Reaction

6. CARBOHYDRATE-AMINO ACID RELATIONSHIPS CONCERNED IN THE INHIBITION OF INDOLE PRODUCTION BY GLUCOSE IN CULTURES OF *ESCHERICHIA COLI*

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The main facts which have been established previously by Happold & Hoyle [1936], and Evans, Handley & Happold [1941; 1942] are that *Escherichia coli* (*Bact. coli*), grown in a complex medium containing glucose and tryptophan, does not contain the tryptophanase enzyme system and that in a simple medium either phenylalanine or tyrosine is necessary to complete the glucose inhibition of the production of the tryptophanase system. A suggestion was made by Evans *et al.* [1942] that the inhibition of indole-production by cells grown in the presence of glucose and phenylalanine is maintained by the metabolism of stored carbohydrate. In this investigation we have attempted to substantiate this suggestion and also to determine the activity of the stereo-isomers of phenylalanine and tyrosine.

EXPERIMENTAL

The bacterial cultures. The Jebbs strain of *Escherichia coli* was used. The medium used for all but one of the investigations had the following composition: *l*-cystine, 1 mg.; glycine, 5 mg.; *dl*-valine, 10 mg.; *dl*-Na glutamate, 50 mg.; Na lactate, 150 mg.; NaCl, 50 mg.; $Na_2HPO_4.12H_2O$, 25 mg.; KH_2PO_4, 3·5 mg.; $MgSO_4.7H_2O$, 3 mg.; $FeSO_4.7H_2O$, 4 μg.; distilled water to 10 ml.; *p*H adjusted to 7·6. Glucose and tryptophan were added as sterile solutions to give a concentration of 1 and 0·003% respectively, whilst phenylalanine, where used, was in a concentration of 0·01%. The total volume of medium used in any particular experiment was 100 ml. Cultures were made in Roux bottles.

Estimation of polysaccharide. A washed suspension of the cells was prepared and its volume noted. A portion of this suspension was taken to obtain the total dry weight of organisms. The polysaccharide content of the remainder was determined by the method of Sahyun [1931]. The following are representative results for the estimation of known amounts of glycogen by this method in our hands:

Glycogen present mg.	Glycogen estimated mg.
8·5	7·4
4·4	3·5
2·2	1·6
1·0	1·1
0·4	0·4

These results indicated that the method was sensitive for small amounts of glycogen of the order of 1 mg. but for larger amounts the degree of recovery was only about 80% and larger amounts of norite had to be used. Since the amount of glycogen estimated in the following experiments was of the order of 1 mg., the method was sensitive enough for our purposes.

Influence of phenylalanine on the metabolism of Esch. coli

(*a*) *Effect of added phenylalanine on oxygen uptake.* Evans *et al.* [1941], using the Van Slyke method for the estimation of amino-N, reported that phenylalanine was not metabolized by *Esch. coli*. This has now been examined with the Barcroft respirometer. 0·5 ml. of a washed suspension of *Esch. coli* grown on casein agar was added to 0·5 ml. of a 0·5% solution of phenylalanine and 4·0 ml. K phosphate buffer (*p*H 7·6) contained in a Barcroft cup. The following results were obtained:

	μl./hr.			
O_2 uptake of cells with phenylalanine substrate	82	86	87	100
O_2 uptake of cells with no substrate	64		63	29

This difference in O_2 uptake, we suggest, corresponds either with the metabolism of phenylalanine or to the acceleration of the metabolism of some substance stored in the cell brought about by the phenylalanine.

(*b*) *Effect of phenylalanine on metabolism of glucose.* With phenylalanine and phenylalanine-free media, the glucose utilization of *Esch. coli* during a period of 24 hr. growth gave the following results:

Phenylalanine-free medium		*dl*-Phenylalanine (0·01%) medium	
Total glucose utilization (mg.)	Glucose utilization (mg./mg. dry bact. wt.)	Total glucose utilization (mg.)	Glucose utilization (mg./mg. dry bact. wt.)
327	3·3	288	4·3
474	7·4	426	7·6
211	4·3	176	4·2

The total glucose metabolized was greater in the absence of phenylalanine and in general total growth was also greater, though this was less marked.

(*c*) *Effect of phenylalanine on polysaccharide storage.* The *dl* and the *l* forms of phenylalanine were used. The results obtained were as follows:

Cells grown in					
Phenylalanine-free medium		*dl*-Phenylalanine (0·01 %) medium		*l*-Phenylalanine (0·01 %) medium	
Polysacc. content (mg./mg. dry bact. wt.)	Total dry bact. wt. (mg.)	Polysacc. content (mg./mg. dry bact. wt.)	Total dry bact. wt. (mg.)	Polysacc. content (mg./mg. dry bact. wt.)	Total dry bact. wt. (mg.)
0·03	19	0·045	20	0·02	27
0·025	92	0·04	112	0·027	118
0·02	53	0·03	31	0·02	27
0·02	48	—	—	0·02	46
0·03	44	0·05	42	—	—
0·01	53	0·03	55	—	—

Thus *dl*-phenylalanine increased the polysaccharide storage of *Esch. coli*, but the naturally occurring *l*-form was inactive in this respect.

(*d*) *Effect of phenylalanine on the metabolism of stored polysaccharide.* Whilst the previous results indicated that there was a difference in the polysaccharide content of the two types of cells, they gave no indication that this polysaccharide was metabolizable. Cells were therefore grown on phenylalanine-free and phenylalanine-containing media, and their O_2 usage estimated in the absence of external substrate by means of a Barcroft respirometer. Typical results were as follows:

Oxygen uptake of cells grown on	
Phenylalanine-free medium μl./hr./mg. dry bact. wt.	*dl*-Phenylalanine medium μl./hr./mg. dry bact. wt.
16·7	41·7
11·0	42·3
29·0	78·0
22·5	36·0
22·5	44·0

A greater amount of some metabolizable substance is present in cells grown in the phenylalanine-glucose medium than in those grown in the phenylalanine-free medium. To confirm that the metabolism of stored polysaccharide did take place during the lag period before indole was produced, when cells grown in a glucose-phenylalanine medium were incubated with tryptophan, the following experiment was performed:

Washed cell suspensions of *Esch. coli* grown in phenylalanine-free and phenylalanine-containing media were prepared. Part of each of these suspensions was incubated with tryptophan and the oxygen consumption of the remainder was determined. The cells grown in the phenylalanine-free medium rapidly produced indole whilst the other cells had a lag period. The O_2 consumption of each of these two types of cells for this lag period was determined. The following are the results obtained:

Oxygen uptake of cells grown on	
Phenylalanine medium μl./hr./mg. dry bact. wt.	Phenylalanine-free medium μl./hr./mg. dry bact. wt.
36·0	18·8
44·0	18·8
40·0	20·0

The average difference between the O_2 uptakes of these two types of cell is 20·8 μl./hr./mg. dry bacterial wt. The amount of glycogen which would require this amount of O_2 for its complete combustion would be approx. 0·025 mg./mg. dry bacterial wt. This amount of glycogen is of the same order as the difference between the polysaccharide content of the cells grown in the two types of media. Therefore we can state that in all probability the increase in polysaccharide content of *Esch. coli* under the influence of phenylalanine corresponds with an increase in the metabolizable polysaccharide stored, and that this polysaccharide is metabolized before the development of a tryptophanase system can take place in the cell.

Influence of tryptophan and tyrosine

(*a*) *Effect of tryptophan on the polysaccharide content.* In this series of experiments the phenylalanine concentration was kept constant at 0·01 %, and the tryptophan content was varied. The following are the results obtained:

Indole production	Tryptophan present in medium %	Polysaccharide content (in duplicate) mg./mg. dry bact. wt.	
–	0·000	0·03	
–	0·003	0·035	0·03
Trace	0·01	0·03	0·025
+	0·02	0·01	0·01

Tryptophan exerts an action antagonistic to that of phenylalanine in stimulating polysaccharide storage in the organism, and evidently the stoichiometric relationship which exists between these two amino-acids in the production of indole in cultures containing glucose is due to this antagonism.

(*b*) *Effect of tyrosine on the polysaccharide content.* The same type of experiment as already described

for phenylalanine was repeated using *dl*- and *l*-tyrosine. The following results were obtained:

Cells grown in					
Tyrosine-free medium		*dl*-Tyrosine medium		*l*-Tyrosine medium	
Polysacc. content (mg./dry bact. wt.)	Total bact. wt. (mg.)	Polysacc. content (mg./dry bact. wt.)	Total bact. wt. (mg.)	Polysacc. content (mg./dry bact. wt.)	Total bact. wt. (mg.)
0·010	40	0·027	54	0·016	74
0·029	39	0·055	26	—	—
0·025	31	0·071	25	0·042	15

Thus results are similar to those obtained with phenylalanine but are less marked.

Influence of mannose

(*a*) *Effect of mannose on the polysaccharide content.* A comparison between the ability of mannose and glucose to allow storage of carbohydrate in *Esch. coli* has been made by determining the effect of replacing glucose in the medium by mannose. Indole was consistently produced in the mannose medium and not in the glucose medium when *dl*-phenylalanine was present. The following are the results obtained:

Polysaccharide content (mg./mg. dry bact. wt.) of cells grown in			
dl-Phenylalanine present		*dl*-Phenylalanine absent	
Glucose medium	Mannose medium	Glucose medium	Mannose medium
0·027	0·013	0·014	0·007
0·034	0·010	0·013	0·010

The range of bacterial dry wt. was from 32 to 82 mg., with the major part falling between 40 and 50 mg. *dl*-Phenylalanine appeared to be unable to increase polysaccharide storage with mannose to any marked extent. There was no evidence of storage with mannose alone.

(*b*) *Metabolism of mannose by* Esch. coli. Ashford & Holmes [1929] showed that in brain there were two mechanisms for glycolysis, one concerned with the breakdown of glycogen and requiring the presence of inorganic phosphate, whilst the other was concerned with the breakdown of glucose and did not require the presence of inorganic phosphate. Subsequently Ashford [1933] showed that whilst the anaerobic breakdown of glycogen and glucose could take place at the same time without any signs of mutual inhibition, mannose and glucose under similar conditions could not be metabolized simultaneously. Thus the conclusion was drawn that the same enzyme system was concerned in the metabolism of both glucose and mannose. In such circumstances both sugars were presumably metabolized by the non-phosphorylating route. In the previous experiments we have shown that glucose in the presence of *dl*-phenylalanine is able to increase the polysaccharide storage in *Esch. coli* and we wondered whether, in this case, the glucose was being metabolized by the phosphorylating route. On the other hand, since mannose is unable to effect such storage it might be metabolized by the non-phosphorylating route. To test this the oxygen consumption of washed suspensions of *Esch. coli* grown on phenylalanine-free glucose medium was determined, using glucose, mannose and a combination of the two as substrates. The concentration of the sugars was 0·5%. To a similar series phenylalanine was added to give a concentration of 0·01%. The results obtained were as follows:

Oxygen uptake of cells in the presence of					
Phenylalanine and			No phenylalanine		
Glucose Q_{O_2}	Mannose Q_{O_2}	Glucose + mannose Q_{O_2}	Glucose Q_{O_2}	Mannose Q_{O_2}	Glucose + mannose Q_{O_2}
11·3	12·1	12·5	12·1	9·8	11·7
7·3	7·6	11·4	11·0	9·0	10·0
10·3	12·3	12·4	14·3	14·5	13·3
5·6	5·9	5·9	—	—	—

Q_{O_2} = μl. oxygen/hr./mg. dry bact. wt.

No apparent summation of O_2 consumption takes place when the cells are acting upon the mixed substrate. Mannose is slightly more readily metabolized than glucose in the presence of phenylalanine and the reverse is true in its absence. The O_2 consumption of the cells metabolizing glucose is apparently greater when phenylalanine is absent from the medium. This conforms with its action in enhancing polysaccharide storage.

DISCUSSION

From the results presented there would appear to be no doubt that the mechanism of the glucose inhibition of the tryptophanase system in *Esch. coli* depends on the ability of the cell to store more polysaccharide than is present in the cell after culture in indole-producing media and that the metabolism of such stored polysaccharide may delay the onset of indole production when such cells are incubated with tryptophan.

Antagonistic actions on the storage of polysaccharide appear to be exerted by tryptophan and phenylalanine, the latter stimulating storage. Thus the suggestion can be made that a labile component of the enzyme systems concerned in the breakdown of tryptophan to indole and in the storage of carbohydrate is common to both and that competition occurs. The role which *dl*-phenylalanine plays in stabilizing the enzyme system so that storage of carbohydrate becomes its main function is obscure.

The 'natural' *l*(−) form appears to be inactive and therefore it is presumably the 'non-natural' *d*(+) form which exerts this action.

Butts, Dunn & Hallman [1938], working with rats, found that *dl*-phenylalanine was very active in the formation of liver glycogen in this animal, although *dl*-tyrosine was found to be inactive. Subsequently Butts, Sinnhuber & Dunn [1941] found that although *dl*-tyrosine was inactive in the formation of liver glycogen, the *l*(−) form caused some storage to occur. Our experiments may indicate that *l*(−)-tyrosine is more active in enhancing polysaccharide storage in *Esch. coli* than is *l*(−)-phenylalanine but in neither case does the *l*(−) form show an activity equal to the activity shown by the *dl* form. The similarities and dissimilarities of the action of phenylalanine and tyrosine on the carbohydrate metabolism of a mammal and of a bacterium open up some interesting questions.

dl-Phenylalanine inhibits to a slight extent the dissimilation of glucose by washed suspension of *Esch. coli*. If the amount of polysaccharide storage depends on a balance between the rate of storage and the rate of dissimilation, then if the latter rate is decreased we shall have increased storage. If we accept the scheme postulated by Hanes [1940] for the metabolism of glycogen by yeast cells, and the generally accepted scheme for the dissimilation of glucose, and apply them to the current problem, then phenylalanine may act either by influencing the equilibrium of the reaction glycogen⇌glucose-1-phosphate, or the dissimilation of the latter. Increase in glycogen storage may be sufficient to explain the decrease in the dissimilation of glucose, or the decrease in the dissimilation of glucose phosphate may be sufficient to explain the increased glycogen.

With regard to the failure of mannose to produce polysaccharide storage in *Esch. coli* under the influence of *dl*-phenylalanine, it would appear true to say that mannose is apparently metabolized by the same route as glucose, and since in this case glucose is capable of forming polysaccharide, it would appear most likely that this route is a phosphorylating one. It should, however, be pointed out that Cori, Schmidt & Cori [1939] prepared an eluate of yeast which together with the addition of adenylic acid and glucose-1-phosphate could produce a polysaccharide. Such a mixture could not, however, produce a polysaccharide from a similar mannose phosphate ester. Thus the conclusion to which we come is that although the mannose may be undergoing metabolism by the phosphorylating route, the enzyme system concerned in polysaccharide formation is unable to form polysaccharide from the mannose-1-phosphate which may be formed.

SUMMARY

1. *dl*-Phenylalanine and *dl*-tyrosine bring about an increased storage of metabolizable polysaccharide in *Esch. coli* when the latter is grown on a medium of known composition containing tryptophan and glucose.

2. The *l*(−) forms of these amino-acids are inactive in bringing about this increased polysaccharide storage.

3. Mannose is unable to increase the polysaccharide storage of *Esch. coli* in the presence of *dl*-phenylalanine, and suggestions have been given to explain this.

4. Tryptophan is antagonistic to *dl*-phenylalanine in its action on polysaccharide storage.

5. Suggestions have been put forward to localize the site of activity of *dl*-phenylalanine, and also to relate the tryptophanase system and the systems concerned in carbohydrate breakdown.

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The Effect of the Plasma Proteins upon the Sedimentation Rate of Human Blood

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Fresh blood which contains an anticoagulant will separate out on standing, leaving a sharp boundary between the clear plasma above and the packed red cells below. The rate of this sedimentation is used as a clinical test, because in the healthy subject it occurs very slowly, while in many pathological conditions it is extremely rapid. From the work of Fahraeus [1929], Tiffeneau & Gysin [1937] and Frazer & Rennie [1941] it is known that the rate of sedimentation is determined by the protein constituents of the plasma, but no clear correlation has yet been found between the observed sedimentation rate and the quantities of the various protein fractions [Alred-Brown & Monroe, 1935; Ropes, Rossmeisl & Bauer, 1939; Frazer & Rennie, 1941]. In this paper the problem has been tackled by building up 'pathological plasmas' with protein fractions isolated from normal plasma and observing the behaviour of normal cells suspended in them.

METHODS

Estimation of sedimentation rate. The following procedure gave consistent results. Blood was collected into a tube containing dry anticoagulant in such amount that 100 ml. of blood contained 0·3–1·0 g. of Na citrate, or 0·5–3·0 g. of heparin. Within 15 min. of withdrawing the blood it was suspended in a Westergren type of tube, giving a column 200 mm. high and 2·8–3·0 mm. in diameter. This was fixed vertically in a rack, and the sedimentation rate expressed in terms of the height in mm. of plasma visible above the erythrocytes at a given time.

Preparation of protein fractions. The material used was normal human plasma which had been citrated and kept for about 6 days at 4°. The salted-out precipitates were dialysed under pressure against 0·85% NaCl until no contaminant salt remained, and the proteins sterilized by Seitz filtration and stored at 4°. Fibrinogen, total globulin, euglobulin, pseudoglobulin and total albumin were precipitated by appropriate concentrations of $(NH_4)_2SO_4$; crystalbumin, globoglycoid and seromucoid were separated as described by Hewitt [1938*a*], and by Rimington & van den Ende [1940], and nucleo-protein by the CO_2 precipitation method of Plimmer [1926]. All these fractions gave Molisch and cystine-S reactions in accordance with the literature. Seroglycoid could not be obtained, and if it does exist in human plasma it will be included in our total albumin fraction, and will not be present as a contaminant in any subsidiary fraction. Hewitt's seroglycoid may be equivalent to our seromucoid.

Our experiments were always duplicated, and usually repeated three or more times on different occasions, using separate batches of the protein in question, and different normal cells. The variation in the results was usually less than ±9%.

RESULTS

Influence of 'purified' plasma proteins on rate of cell sedimentation

(*a*) *Influence of mode of preparation of total globulin fraction on its action on sedimentation.* Fahraeus [1918], and later Tiffeneau & Gysin [1937], showed that if cells were suspended in solutions of protein fractions isolated from plasma, the sedimentation rates obtained were in the order, fibrinogen > globulin > albumin. Before attempting to repeat these results, experiments were carried out to ascertain whether the treatment undergone by a protein fraction during isolation affected its sedimentation-influencing properties. The results obtained with the total globulin fractions separated from portions of the same batch of normal plasma by three different methods (Table 1) showed substantial differences, despite the fact that the pseudoglobulin/euglobulin ratios were the same (4/1) for all three preparations.

Table 1. *Rate of sedimentation of human erythrocytes in the presence of serum globulin prepared by different methods*

(Total globulin conc. = 3 g./100 ml. 0·85% NaCl. Cell vol. = 20%.)

	Total globulin fractions prepared by precipitation by		
	Sat. with NaCl	Sat. with $MgSO_4$	½-sat. with $(NH_4)_2SO_4$
Sedimentation after 30 min. (mm.)	10	15	5

(*b*) *Action of single plasma proteins prepared as described under Methods.* We repeated the experiments of Fahraeus [1918] and of Tiffeneau & Gysin [1937], using a greater range of plasma protein fractions. From the results shown in Table 2, it will be seen that the most rapid sedimentation rates are obtained with fibrinogen and euglobulin. The question arose whether the 'slower' proteins, such as total albumin, when mixed with a 'fast' protein, merely reduced the sedimentation rate by acting as a diluent, or if the resulting sedimentation rate was the mean of those for the two separate proteins.

Table 2. *Rate of sedimentation of human erythrocytes suspended in 'pure' solutions of normal plasma protein fractions*

(Protein conc. = 3 g./100 ml. 0·85% NaCl. Cell vol. = 20%.)

Protein	Average sedimentation (mm.) after 1 hr.	4 hr.	24 hr.	No. of exp.
Fibrinogen	100	150	150	3
Total globulin:				
(*a*) Pseudoglobulin/euglobulin ratio = 4:1	4	22	100	2
(*b*) Pseudoglobulin/euglobulin ratio = 1·6:1	14	20	130	2
Euglobulin	20	50	70	3
Pseudoglobulin	2	16	50	4
Total albumin	1·5	8	26	3
Crystalbumin	2	10	40	2
Globoglycoid	2	5	26	1
Seromucoid	3	7	37	1
Nucleoprotein	3	8	40	2

(*c*) *Interaction of two plasma proteins.* For experiments with fibrinogen three tubes were set up containing (*a*) fibrinogen (3 g./100 ml.); (*b*) the same fibrinogen solution diluted with an equal volume of saline, and (*c*) the fibrinogen solution diluted with an equal volume of a 3 g./100 ml. solution of total albumin. The results (Table 3) show that albumin

Table 3. *Rate of sedimentation of human erythrocytes suspended in solutions of fibrinogen diluted with saline or albumin*

(Fibrinogen and albumin sol. = 3 g./100 ml. 0·85% NaCl. Cell vol. = 20%. Results mean of 5 experiments.)

	Equal vol. of: Fibrinogen and fibrinogen	Fibrinogen and 0·85% NaCl	Fibrinogen and albumin
Sedimentation after 1 hr. (mm.)	100	40	6·0

does not act simply as a diluent; if the sedimentation rate in the mixture were the mean between that in fibrinogen (100 mm.) and that in albumin (1·5 mm.), the reading in the third tube should have been 50·7 mm. Actually the albumin inhibits the action of the fibrinogen so strongly that the reading is scarcely more than that given by a 3% solution of albumin alone. The action of other proteins upon fibrinogen was therefore studied, in order to ascertain whether this inhibitory action was peculiar to albumin.

The series of experiments in Table 4 shows that globulin slightly accelerates the sedimentation rate in fibrinogen solutions, and that this effect is due to the euglobulin fraction. The inhibiting effect of the albumin is caused by globoglycoid, while crystalbumin and seromucoid have hardly more effect than a mere saline dilution. The effect of nucleoprotein

Table 4. *Sedimentation of human erythrocytes suspended in solutions of fibrinogen mixed with equal volumes of another protein fraction*

(Protein conc. = 3 g./100 ml. 0·85% NaCl.)

Protein mixture	Mean sedimentation at end of 1st hr. mm.	No. of exp.
Fibrinogen and saline	40	2
Fibrinogen and total globulin (pseudoglobulin/euglobulin ratio = 4:1)	60	2
Fibrinogen and euglobulin	75	2
Fibrinogen and pseudoglobulin	30	3
Fibrinogen and total albumin	4·6	4
Fibrinogen and crystalbumin	50	3
Fibrinogen and globoglycoid	10	2
Fibrinogen and seromucoid*	70	1
Fibrinogen and nucleoprotein	1·0	3

* Seromucoid 1·8%.

is surprising, for after 24 hr. the fall is only 3 mm. Its normal concentration is low (0·003 g./100 ml. plasma), and since it is insoluble at 20% sat. $(NH_4)_2SO_4$, it will occur as a contaminant in the fibrinogen fraction.

These results suggest that the interaction of proteins in the plasma has a great influence upon erythrocyte stability. The effect of combining any two protein fractions in equal proportions has been worked out in some detail, and the corresponding saline dilutions given for comparison. The results tabulated in Table 5 are expressed as the fall at the end of 3 hr. with a 20% volume of fresh normal cells. The 3rd hr. reading is presented here because the 'slower' proteins fail to show significant differences at 1 hr., and the 'faster' have all reached the terminal packing stage by the 5th hr. To find the sedimentation for, say, a combination of fibrinogen and euglobulin, take the horizontal fibrinogen row of figures and follow it along until it intersects the vertical column for euglobulin. The reading in this square is 142 mm., which represents the average 3rd hr. fall for this combination. Table 5 shows the action of the proteins upon fibrinogen and also the inhibitory effect of total albumin upon the globulins, which is not so great in proportion as with fibrinogen. Nucleoprotein gives a greater sedimentation rate alone than when mixed with fibrinogen. We can give no explanation of this, but the experiment was repeated three times with the same result. The interaction between the 'slower' proteins is uninteresting. The figures show the effect of two proteins only in solution: in plasma, however, all the fractions are coexistent, and as we have shown that albumin inhibits fibrinogen, it seems likely that albumin will also inhibit the euglobulin which, if alone, would accelerate the fibrinogen, and thus exercise a double inhibition upon fibrinogen.

Table 5. *Sedimentation of human erythrocytes suspended in mixtures containing equal volumes of two plasma proteins*

(Protein conc. = 3 g./100 ml. 0·85% NaCl. Results given as mean of 2–4 experiments.)

	Mean sedimentation after 3 hr. mm.									
	Nucleo-protein	Sero-mucoid	Globo-glycoid	Cryst-albumin	Total albumin	Pseudo-globulin	Euglo-bulin	Total globulin	Fib-rinogen	Saline
Saline	—	—	3·5	—	5	7	10	7	97	3
Fibrinogen	3	130	40	100	10	60	142	130	150	97
Total globulin	—	—	6	7	7	9	20	11	130	7
Euglobulin	—	—	5	7	2	10	25	20	142	10
Pseudoglobulin	—	—	3	35	5	15	10	9	60	10
Total albumin	—	—	3	3	5	5	2	7	10	5
Crystalbumin	—	—	2	6	3	35	7	7	100	—
Globoglycoid	—	—	3	2	3	3	5	6	40	3·5
Seromucoid	—	5	—	—	—	—	—	—	130	—
Nucleoprotein	5	—	—	—	—	—	—	—	3	—

(*d*) *Interaction of three or more plasma proteins.* This type of interaction was worked out in three stages:

(1) Fibrinogen altered: Constant globulin and albumin.

(2) Globulin and its fractions altered: Constant fibrinogen and albumin.

(3) Albumin and its fractions altered: Constant fibrinogen and globulin.

The effect of fibrinogen upon sedimentation rate was studied by comparing the fall of cells in plasma with that in serum from the same person, part of the specimen being collected with oxalate and part being allowed to clot for serum. The oxalated cells were centrifuged, washed in saline, and resuspended in the plasma and the serum. Table 6 shows that

Table 6. *Comparison of sedimentation rates of human erythrocytes suspended in plasma and in serum from the same blood*

(Five experiments performed.)

	Erythrocytes suspended in	
	Plasma	Serum
Normal blood, cell vol. reduced to 20%. Sedimentation at 7th hr. (mm.)	15	6
Pathological blood, cell vol. unaltered. Sedimentation after 1 hr. (mm.)	60	6

although some sedimentation can take place in the absence of fibrinogen, it plays the chief part, and therefore its acceleration or inhibition will have a great effect upon sedimentation. Oakley [1938] was able to add normal blood of known fibrinogen content to blood which completely lacked fibrinogen, and found that the relation between increased sedimentation rate and fibrinogen content was practically linear.

The second and third types of reaction were studied by constructing artificial plasma from a mixture of single protein fractions. In order to test the accuracy of this method, sedimentation rates on normal and pathological blood were carried out parallel with analyses on the plasma, and records of cell size and cell volume kept. From these data artificial plasma was built up, the correct volume of fresh normal cells suspended in it, and the sedimentation rate determined. The total protein percentages were often lower than in the original, because it was found difficult to obtain all the individual fractions sufficiently concentrated, but the ratio of the component fractions was maintained. The protein solutions must be in good condition, for a denatured batch of, say, fibrinogen will invalidate the whole experiment. The results of imitating normal and pathological blood are shown in Tables 7 and 8.

Table 7. *Sedimentation of human erythrocytes suspended in natural or artificial normal plasma*

	Composition of natural normal plasma proteins as g./100 ml.	Composition of artificial normal plasma g./100 ml.
Fibrinogen	0·3	0·24
Globulin	2·7	2·16
Albumin	4·0	3·2
Total protein	7·0	5·6
Cell vol. (%)	40·0	40·0
Cell diam. (μ)	7·1	7·1
Sedimentation after 1 hr. (mm.)	4·0	3·0

From these two experiments we conclude that an artificial mixture of normal proteins does behave similarly to natural pathological plasma, in its effect upon sedimentation.

The globulin fractions were studied on the basis of the tuberculous plasma analysis given in Table 8. It will be seen that the fibrinogen and albumin were maintained constant and total globulin first replaced by euglobulin, and then by pseudoglobulin.

Table 8. *Sedimentation of human erythrocytes suspended in natural and artificial tuberculous plasma*

	Composition of plasma (proteins as g./100 ml.)			
		Artificial plasma containing		
	Natural plasma	Total globulin	Eu-globulin	Pseudo-globulin
Fibrinogen	0·6	0·6	0·6	0·6
Total globulin	3·32	3·2*	—	—
Euglobulin	2·15	—	3·2	0
Pseudoglobulin	1·17	—	0	3·2
Albumin	3·6	3·6	3·6	3·6
Total protein	7·52	7·4	7·4	7·4
Cell vol. (%)	40·0	40·0	40·0	40·0
Sedimentation after 1 hr. (mm.)	94·0	90·0	80·0	26·0

* Prepared by precipitation by $MgSO_4$.

Because euglobulin slightly accelerates the sedimentation rate of fibrinogen (Table 4) we consider that the artificial plasma with euglobulin ought to have shown a greater sedimentation rate than total globulin, and attribute the slightly lower reading to a poor batch of the protein. The inhibitory action of the pseudoglobulin is, however, clear enough. Some months after this experiment was performed, a case of tuberculosis became available in which the sedimentation rate was low and the pseudoglobulin high, while the disease was sufficiently advanced for the sputum to show great numbers of tubercle bacilli. The results were as follows:

Plasma protein content		
Protein	Conc. g./100 ml.	
Fibrinogen	0·5	
Euglobulin	0·1	Cell vol. = 36%
Pseudoglobulin	2·3	Sedimentation (1 hr.) = 0·5 mm.
Albumin	4·49	

This analysis may well be compared with that for plasma and the artificial plasma containing pseudoglobulin (Table 8).

A typical nephritic blood was constructed in order to study the effect of the component fractions of albumin. This was chosen because there is much known about the albumin changes in nephritis and nephrosis. The fibrinogen and globulin were kept constant throughout; normal total albumin was used first to see if a typically rapid nephritic sedimentation could be obtained.

The sedimentation (Table 9) is not so great as one would expect in natural blood with the same composition. The total albumin was then replaced by globoglycoid and then by crystalbumin. The plasma containing globoglycoid (Table 9) gave an extraordinarily slow sedimentation, and the crystalbumin mixture caused a far more rapid fall than could be obtained in real nephritic blood. The interesting point is that the component fractions of albumin have a remarkably different effect upon the sedimentation rate. Unfortunately, seromucoid could not be prepared in sufficiently concentrated solution for use in these experiments.

Table 9. *Sedimentation of human erythrocytes suspended in artificial nephritic plasma*

	Composition of artificial plasma (proteins as g./100 ml.) containing		
	Total albumin	Globo-glycoid	Cryst-albumin
Fibrinogen	0·32	0·26	0·32
Globulin	3·2	2·6	3·3
Total albumin	1·7	0·0	0·0
Crystalbumin	0·0	0·0	1·6
Globoglycoid	0·0	1·3	0
Total protein	5·2	4·2	4·2
Cell vol. (%)	20·0	20·0	20·0
Sedimentation after 1 hr. (mm.)	15·0	1·0	145·0

Hewitt [1938*a*] analysed nephritic serum and found the crystalbumin fraction to be far below normal, while the globulin and seroglycoid (equivalent to seromucoid?) were not strikingly different; no mention, however, is made of globoglycoid. If crystalbumin is reduced in real nephritic blood, one would expect, from these experiments, a slower sedimentation rate. Unfortunately, there is no convenient method of analysing the albumin components on a small sample of blood, and our results do not tally with his findings.

DISCUSSION

From this series of experiments we conclude that the power of sedimentation of blood is not controlled by the absolute concentration of either the total plasma proteins or the protein fractions, but by *the inhibition of one protein by another*. Thus it is quite possible to have a high fibrinogen content and low sedimentation rate due to increase of pseudoglobulin at the expense of the euglobulin, while the total globulin concentration remains stationary; or an increase of globoglycoid over crystalbumin can inhibit the action of the globulins without inducing any abnormality in the gross protein fractions. This concept probably explains why Alred-Brown & Monroe [1935] and Ropes *et al.* [1939] found no relation between the concentration of any one protein fraction and sedimentation rate, while Frazer & Rennie [1941] found decrease of albumin and, to a lesser extent, increase of fibrinogen, associated with rise of sedimentation rate, while the globulins had no appreciable effect.

Our experiments suggest that when the blood sedimentation rate is raised, there is no alteration in the chemical structure of the proteins. A possible exception to this is the Bence-Jones protein [Devine, 1941]. Geottsch [1936] found that nephritic total globulin was immunologically different from the normal, and Alving [1936] found changes in the cystine content of total albumin in nephrosis. Hewitt [1938*b*] later showed that in both cases the individual fractions of globulin or albumin give normal chemical and immunological reactions, and the altered ratios are responsible for the abnormality of the plasma.

SUMMARY

1. The proteins from normal plasma were separated into nine fractions, and the rate of fall of a 20% vol. of erythrocytes in 3% solutions of the proteins during 1 hr. was found to range from 100 mm. for fibrinogen to 1·5 mm. for total albumin.

2. The fractions were combined in equal amounts using 39 pairs. The action of the 'faster' fractions, fibrinogen and euglobulin, is inhibited by the 'slow' nucleoprotein and globoglycoid.

3. Artificial pathological plasmas built up from these fractions gave sedimentation rates similar to those for the comparable natural material. With artificial tuberculous plasma it was found that by altering only the component globulin fractions, the sedimentation rate could be changed from 94 to 26 mm.; and with artificial nephritic blood, substitution of total albumin by crystalbumin altered the rate from 15·0 to 145 mm., while substitution of the total albumin by globoglycoid changed it from 15·0 to 1·0 mm.

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Phosphorolysis and Synthesis of Glycogen in Animal Tissues

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The mechanism of glycogenolysis in muscle and liver is described by Cori, Cori & Schmidt [1939] as follows:

(1) Glycogen + phosphate ⇌ glucose-1-phosphate (catalysed by phosphorylase and adenylic acid).

(2) Glucose-1-phosphate ⇌ hexose-6-phosphate (catalysed by phosphoglucomutase and Mg^{++}).

(3) Hexose-6-phosphate ⇌ glucose + phosphate (catalysed by phosphatase in liver).

(3*a*) Hexose-6-phosphate ⇌ hexose-1:6-diphosphate → lactic acid (in muscle).

These reactions are responsible for glucose formation in liver, and are the first steps in glycolysis in muscle. Glycogen resynthesis too is probably explained by the same reactions [Cori & Cori, 1941]. It seemed of interest, therefore, to study the distribution of the enzymes concerned, in various tissues at various stages of development, and to compare the enzymic activities with the rate of metabolism of glycogen in the tissue concerned. The further question was also investigated in this connexion whether insufficiency of one or more of the designated enzymes is the cause of metabolic disturbances in certain pathological conditions.

A separate series of experiments dealt with some properties of phosphorylase, and in particular with its inhibition by glucose. This inhibition was first observed by Lehmann [1938] who suggested its possible physiological importance. It may be responsible for the homeostatic mechanism described by Soskin, Essex, Herrick & Mann [1938].

METHODS

Preparation of phosphorylase

Phosphorylase was prepared according to Cori *et al.* [1939] by adsorbing the enzyme of tissue extracts on $Al(OH)_3$ and eluting with Na glycerophosphate. It was found, however, that C_γ $Al(OH)_3$ prepared according to Willstätter, Kraut & Erbacher [1925] frequently yielded inactive enzyme preparations. With the C_β variation, on the other hand, active preparations were regularly obtained, and remained active for long periods provided the precipitate had been washed only once with NH_4OH instead of twice.

Estimation of enzymic activity

(*a*) *Phosphorylase activity* was estimated on the basis of the amount of glycogen synthesized from glucose-1-phosphate in the presence of the enzyme. A mixture of 1 vol. enzyme solution and ½ vol. of a solution containing 3% glucose-1-phosphate and 1½% glycogen in *N*/10 H_2SO_4 is incubated at 25° for various periods. In some cases 0·25 mg. adenylic acid is added per ml. of the mixture. The extent of glycogen synthesis was determined by measuring the amount of inorganic phosphate liberated.

(*b*) *Phosphoglucomutase activity* was estimated as the decrease in easily hydrolysable phosphate (10 min. hydrolysis with *N*/1 HCl at 100°) when glucose-1-phosphate is added to 0·3 g. of tissue brei suspended in 2 ml. of bicarbonate Ringer containing *M*/40 NaF, and incubated at 37° with continuous shaking. The bicarbonate Ringer is composed of 4 ml. 1·15% KCl, 1 ml. 0·8% $MgSO_4$, 100 ml. 0·9% NaCl and 60 ml. 1·3% $NaHCO_3$. Before use the solution is saturated with CO_2.

(*c*) *Glycogen phosphorolysis.* The conversion of glycogen into hexose-6-phosphate was measured by estimating the disappearance of inorganic phosphate when 0·3 g. of tissue brei was incubated with 1% glycogen and *M*/40 NaF in phosphate-Ringer at 37° with continuous shaking. The phosphate-Ringer is similar in composition to the bicarbonate-Ringer except that phosphate buffer *M*/10 of *p*H 7·4 is used instead of bicarbonate.

(*d*) *Estimation of phosphate.* Phosphate was estimated according to Lohmann & Jendrassik [1926] after the reactions had been stopped by the addition of 1 ml. 20% $CCl_3.COOH$. In the case of glycogen synthesis from glucose-1-phosphate the acid solution is immediately diluted to avoid splitting of the ester by acid.

RESULTS

Distribution of the phosphorolytic system

Phosphorylase was found in almost all tissues (Fig. 1). Organs with a highly active glycogen metabolism, such as muscle, liver and heart, however, give much more active phosphorylase preparations than organs which are devoid of, or have only a slight glycogen metabolism, as, for example, lungs, skin, testes, intestine, spleen, adipose tissue and placenta. Kidney and brain occupy an intermediary position as regards both phosphorylase activity and glycogen metabolism.

The distribution of phosphoglucomutase and glycogen phosphorylase is generally parallel to the

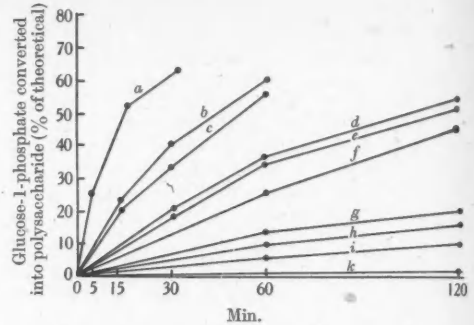

Fig. 1. Glycogen synthesis from glucose-1-phosphate by phosphorylase in tissue brei. *a*, muscle; *b*, heart and subcutis; *c*, liver; *d*, kidney; *e*, brain; *f*, tumour; *g*, lung and intestine; *h*, skin, testes and spleen; *i*, adipose; *k*, placenta.

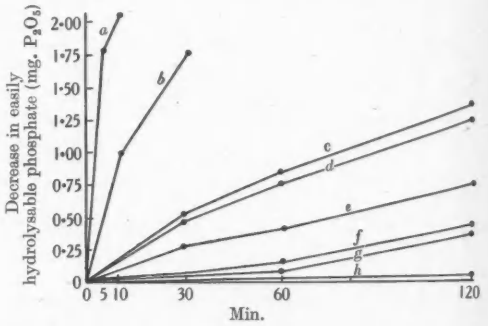

Fig. 2. Conversion of glucose-1-phosphate into hexose-6-phosphate in tissue brei. *a*, muscle; *b*, heart; *c*, skin; *d*, brain; *e*, spleen; *f*, lung; *g*, tumour; *h*, intestine, adipose and testes.

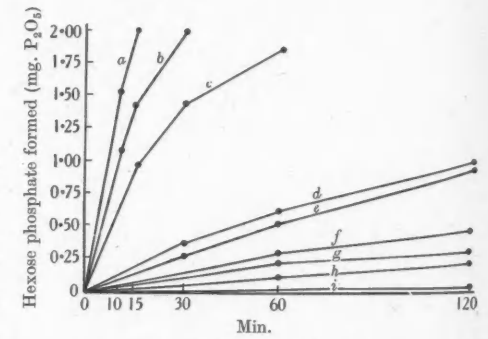

Fig. 3. Phosphorolysis of glycogen in tissue brei. *a*, muscle; *b*, liver; *c*, heart; *d*, brain; *e*, kidney; *f*, skin; *g*, lung; *h*, tumour; *i*, intestine, testes, adipose, pancreas and placenta.

intensity of glycogen metabolism. Yet several exceptions were observed (Fig. 2, 3). Tumour tissue (sarcoma), though it is not generally classed as a glycogen-metabolizing tissue, displayed a high phosphorylase activity. The phosphorolytic breakdown of glycogen by this tissue is, however, very slow as compared with that of other tissues. This apparent discrepancy is due to the low phosphoglucomutase activity of tumour tissue.

Glycogen metabolism of subcutaneous tissue

A further apparent disagreement occurs with subcutaneous tissue. This tissue is one of the most active as regards the phosphorylase activity, but was not previously known to possess any high glycogen-metabolizing capacity. It was found, however, that this tissue has a regular and high glycogen metabolism (Table 1), and that subcutaneous tissue from normal rats contains quantities of glycogen comparable with those of muscle tissue. This glycogen store is readily mobilized during fasting and thus becomes depleted. On administration of adrenalin the glycogen content drops. This decrease was observed with doses of adrenalin causing an elevation of blood sugar, as well as with low doses having no marked effect on the blood-sugar level. Sugar administration by stomach tube caused a marked elevation of the glycogen content of the subcutaneous tissues.

Table 1. *Glycogen content of subcutaneous tissues*

Treatment of animal	No. of exp.	Blood sugar (mg./100 ml.)	Average glycogen content of subcutaneous tissues %
Normal rats	24	121	0·27
1–2 days' fast	12	99	0·06
0·03 mg. adrenaline 1–2 hr. previously	5	175	0·06
0·005–0·01 mg. adrenaline 1–2 hr. previously	5	125	0·05
6 ml. 25% glucose *per os*, in addition to regular diet	8	110	0·50

When rats received a normal, carbohydrate-rich diet after prolonged undernutrition (causing 20% loss of body wt.), an exceptionally high glycogen content was found in the subcutaneous tissues on the 1st day after recommencement of the normal feeding. After 7 days on the usual carbohydrate-rich diet the glycogen content is again normal. In Table 2 the results with subcutaneous tissue are compared with those for glycogen in adipose tissue and in liver, under similar conditions [Tuerkischer & Wertheimer, 1942].

Subcutaneous tissue may thus be regarded as a glycogen-metabolizing tissue, and appears to constitute a supplementary glycogen store for the body.

The development of the phosphorolytic system

It is known, largely from the work of Needham and his collaborators [Needham & Lehmann, 1937; Needham & Nowinski, 1937], that embryonic tissue acts preferably on glucose, and only very slowly on glycogen, to form lactic acid. Chick embryo is rarely able to phosphorylate glycogen. The glycogenolytic system in various organs develops only with advancing age. Experiments were undertaken in order to identify the rate-limiting component of the glycogenolytic system, and to clarify the course of its development.

In muscle tissue from rats up to 10 days of age no phosphorylase activity could be detected (Table 3). This lack of activity is not due to the lack of the coenzyme adenylic acid, since even after addition of optimal amounts of coenzyme only an insignificant glycogen synthesis took place. As Table 3 shows, muscle phosphorylase activity rises rapidly after the 10th day and is substantial by the 14th day. At this stage the amount of adenylic acid present in muscle is the limiting factor, since the addition of this substance caused an acceleration of glycogen synthesis which is never observed in adult muscle tissue.

Table 3. *Glycogen synthesis in muscle tissue from young rats*

	Glucose-1-phosphate converted into glycogen in 1 hr. (% theoretical)	
Age in days	Without addition of adenylic acid %	With addition of adenylic acid %
10	0	5
14	26	45
20	25	52

Table 2. *Glycogen content of subcutaneous tissues after normal feeding of rats previously subjected to prolonged undernutrition*

	Glycogen content of tissue (%)							
	After 7 days' undernutrition wt. loss = 20%	Days after re-feeding with carbohydrate-rich diet						
Tissue		1	2	3	4	5	6	7
Subcutaneous tissue	0·07	0·58	0·92	0·61	0·51	0·50	0·55	0·32
'White' adipose tissue	0·00	0·55	0·90	0·50	0·23	Trace	0·00	0·00
'Brown' adipose tissue	0·00	1·85	2·40	1·80	0·27	Trace	0·00	0·00
Liver	0·40	7·2	6·3	6·0	3·5	3·1	—	—

The rate of development of the glycogenolytic system in brain is quite different from that in muscle. The activity of the various components of the phosphorolytic system in relation to the activity of normal adult tissue—taking the last as 100—is shown in Fig. 4. It can be seen that in contrast to

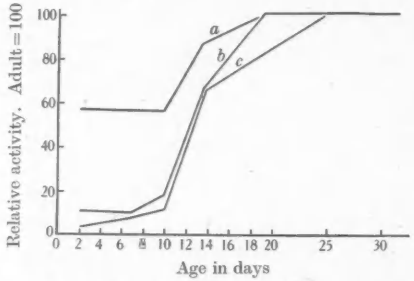

Fig. 4. Phosphorolysis, glycogen synthesis, and phosphoglucomutase action in brain of rats at different ages. *a*, glycogen synthesis; *b*, phosphorolysis; *c*, phosphoglucomutase.

the results with muscle, the glycogen-synthesizing capacity is already present immediately after birth to the extent of about 50% of the adult value. Nevertheless, no phosphorolytic breakdown of glycogen takes place at this age. This is accounted for by the complete lack of phosphoglucomutase activity. Thus, the glucomutase and not the phosphorylase is the limiting factor in glycogenolysis in the brain of young rats. As Fig. 4 shows, the phosphorolytic capacity of brain parallels the development of the mutase activity. Following an initial period of a very slow development up to the age of 10 days, the mutase begins to develop quickly, and at 14 days about 70% of the adult activity is reached. After this age the results become less uniform. In general, the adult value for both mutase and glycogen phosphorolysis is reached at an age between 20 and 30 days. The phosphorylase system develops independently. Its relative activity was always higher than other systems examined. Hence, it can at no stage be regarded as the limiting factor of glycogen metabolism during development.

It remained to be established whether the development of the enzymic activities is due to the development of new kinds of cells or is the result of formation of new enzymes in cell types already present at birth. For this purpose the activity of white and of grey brain matter was compared. The results (Table 4) show that no significant difference in glycogen phosphorolysis can be observed in white and grey matter of brain. The development of the phosphorylase system thus seems to take place in all parts of the brain and not be specially due to the development of the grey matter.

Table 4. *Phosphorolysis of glycogen in white and grey matter of rabbit brain*

Tissue	Hexose phosphate formed (mg. P_2O_5) in 30 min.	60 min.	120 min.
(1) White matter	0·50	0·85	1·00
Grey matter	0·48	0·65	0·95
(2) White matter	0·60	0·85	0·98
Grey matter	0·64	0·93	1·10
Nerve fibre	0·48	0·63	0·70

Phosphorolysis in pathological conditions

Since it had been shown that a parallelism exists between the capacity of an organ to metabolize glycogen and the activity of its phosphorolytic system, it was of interest to determine whether a deficiency in the glycogen phosphorolytic system could be found in pathological cases in which glycogen metabolism is disturbed.

The effect of liver damage produced by various methods was first examined.

Table 5. *Phosphorolysis of glycogen in animals subjected to liver damage*

Animal	Experimental treatment	Initial glycogen content %	Fat content %	Hexose phosphate formed in brei (mg. P_2O_5) during 15 min.	30 min.	60 min.
(*a*) Liver tissue:						
Guinea-pig	None	2·4	4	1·32	2·05	2·45
,,	CCl_4	0·4	8	0·70	1·45	1·60
,,	,,	0·3	9	0·70	1·43	1·63
,,	,,	0·0	8	0·48	0·87	1·23
,,	,,	0·0	9	0·42	0·78	1·10
,,	P	2·0	5	0·95	1·50	2·25
,,	,,	Trace	8	0·62	1·24	1·82
,,	,,	,,	11	0·48	0·55	0·86
,,	,,	,,	10	0·54	0·68	0·94
Rabbit	None	2·0	4	1·10	1·82	2·32
,,	CCl_4	0	7	0·30	0·77	0·93
,,	,,	0	9	0·00	0·00	0·40
,,	$CHCl_3$	0·4	9	0·25	0·53	0·85
,,	,,	0·5	8	0·37	0·64	0·98
,,	Phlorrhizin (fasting)	0·0	8	0·82	1·36	1·67
,,	,,	0·2	9	0·75	1·24	1·75
(*b*) Muscle tissue:						
Guinea-pig	None	—	—	1·45	2·03	2·40
,,	CCl_4	—	—	1·67	2·50	2·55
,,	,,	—	—	1·33	2·05	2·18
,,	,,	0	8	1·45	2·10	2·40
,,	,,	0	9	1·30	2·08	2·43
,,	P	2·0	5	1·80	2·60	2·80
,,	,,	Traces	8	2·44	2·74	2·94
,,	,,	,,	11	1·68	2·30	2·56
,,	,,	,,	10	1·63	2·00	2·40

It is evident from the data (Table 5) that livers damaged by poisons such as CCl_4, P and $CHCl_3$ show impaired glycogen phosphorolysis breakdown. The fat and glycogen contents of liver served as measures of liver damage. It may be seen that when the liver fat has been raised above 8%, with low glycogen content, by the administration of the above poisons, the phosphorolytic capacity of the liver is far below normal. When fat accumulation was induced in the liver without damage, by prolonged fasting and phlorrhizin treatment, no comparable loss of phosphorolytic capacity was observed.

The diminution in phosphorolytic activity does not seem to be due to inactivation of phosphorylase, since, even in cases of almost complete lack of phosphorolytic activity, phosphorylase preparations from the same liver synthesize glycogen normally. The diminution may be due to an inactivation of the phosphoglucomutase. Further proof of this assumption is not available, since the estimation of phosphoglucomutase in liver is not practicable, liver phosphatase being active even in the presence of high concentrations of fluoride. In liver brei glucose-1-phosphate is split to give inorganic phosphate without the accumulation of hexose-6-phosphate.

Phosphorolysis in muscle tissue from adrenalectomized and thyroidectomized rats

Phosphorolysis was examined in muscles of adrenalectomized rats maintained in good condition by Rubin-Krieck's salt solution. Rats were used 10–30 days after adrenalectomy, and estimations were carried out after 10–30 min. incubation of the brei. Phosphorolysis in muscle of such rats was either normal or slightly diminished. The results agree with those of Holmes & Lehmann [1940] who, working on liver, found no loss of phosphorolysis activity in minced liver of adrenalectomized rabbits. They contradict those of Verzar & Montigel [1942], who found that phosphorolysis in the muscles of adrenalectomized rats was almost completely abolished when short incubation times (up to 30 min.) were used. It has, however, been shown by Holmes & Lehmann [1940] that the glycogen synthesis from glucose by rabbit-liver slices is lowered when the animals used were adrenalectomized. Bendall & Lehmann [1942] obtained the same result with rat-liver slices; they could restore the normal activity by a previous injection with extract from the suprarenal cortex.

Thyroidectomized rats also showed normal phosphorolytic power. The rats were maintained before and after operation on a diet containing fresh milk. Similar results were obtained throughout an interval of 1–4 months after operation.

In thyrotoxicosis also, phosphorolytic activity was found to be normal.

Properties of the phosphorylase system

(*a*) *Heat inactivation.* The phosphorylase of muscle of rats and rabbits is highly thermolabile. Heating to 60° for 5 min. causes complete destruction of the enzyme. At 55°, 20 min. were needed for complete destruction, whereas at 50° even after 60 min. some activity was preserved.

(*b*) *Ultra-violet irradiation.* Phosphorylase from rabbit muscle was irradiated by a mercury lamp at a distance of 50 cm. for 30 min. The temperature was not allowed to rise above 30°. Controls were maintained under the same conditions but covered to prevent penetration of ultra-violet light. The irradiation caused partial or complete inactivation of phosphorylase activity.

(*c*) *Inhibition by glucose.* In confirmation of the results of Lehmann [1938], Gill & Lehmann [1939], Lehmann [1939] and of Cori & Cori [1940], glycogen phosphorylation was found to be inhibited by glucose. A concentration of glucose of about 2% caused almost complete inhibition. On the other hand, in contradiction to Cori's results, glucose inhibition was found to be counteracted by adenylic acid, as is shown in Tables 6 and 7.

Table 6. *Inhibition by glucose of phosphorolysis in incubated muscle brei; influence of adenylic acid in counteracting this inhibitory action of glucose*

Material added	Hexose phosphate formed in 15 min. mg. P_2O_5	Observed inhibition of hexose phosphate formation (% of max. possible)
None	2·13	0
Glucose (initial conc. = 2%)	0·35	84
Adenylic acid (3 mg./ml.)	1·97	0
Adenylic acid (3 mg./ml.) plus glucose (initial conc. = 2%)	1·35	30

Na lactate, as well as Na pyruvate, caused only partial inhibition of glycogen synthesis even at concentrations as high as 2%. Adenosine-5-phosphoric acid (referred to here as adenylic acid) cannot be replaced by adenosine-3-phosphoric acid as a counteractant of glucose inhibition.

The discrepancy between these results and those of Cori is explained by the fact that Cori used lower concentrations of glucose. As is shown in Table 7, when concentrations of 0·3% glucose are used no influence of adenylic acid is detected. Addition of adenylic acid causes the reactivation of the enzyme only to a certain degree, so that after addition of adenylic acid the same activity is found with 2% as with 0·3% of glucose.

The inhibition of phosphorylase by glucose cannot be explained simply by competition between glucose

Table 7. *Inhibition by glucose of glycogen formation in muscle eluate; influence of adenylic acid in counteracting this inhibitory action of glucose*

Incubation period (min.)	Material added: Glucose (initial conc. %)	Material added: Adenylic acid (mg. added to 1 ml. eluate)	Formation of glycogen from added glucose-1-phosphate (% of theoretical)	Inhibition of glycogen formation from glucose-1-phosphate (% of max. possible)
10	0·0	0·0	44	0
	2·0	0·0	1	98
15	0·0	0·0	46	0
	2·0	0·0	7	85
	2·0	1·5	30	35
	2·0	(1·5*)	8	83
	0·0	0·0 plus Na lactate (initial conc. = 2%)	34	26
	0·0	0·0 plus Na pyruvate (initial conc. = 2%)	24	48
20	0·0	0·0	53	0
	2·0	0·0	3	95
20	0	0	25	0
	0·3	0	15	40
	2·0	0	2	92
	0	1·5	53	0
	0·3	1·5	35	34
	2·0	1·5	35	34

* Adenosine-3-phosphate.

and glucose-1-phosphate for the enzyme. Further proof for this is given by experiments with potato phosphorylase. This phosphorylase, like other phosphorylases from plants, is not activated by adenylic acid. Competition between glucose and glucose-1-phosphate should exist in this system as well as in animal phosphorylases, and phosphorylase action should be inhibited. If, however, glucose inhibition is connected with activation by adenylic acid, no inhibition by glucose should be observed with potato enzyme. The results in Table 8 show that the latter alternative applies.

Table 8. *Inhibition by glucose of glycogen formation in eluate of potato juice*

Incubation period (min.)	Glucose added (initial conc. %)	Formation of glycogen from added glucose-1-phosphate (% of theoretical)	Inhibition of glycogen formation from glucose-1-phosphate (% of max. possible)
15	0·0	43	0
	2·0	41	5
30	0·0	58	0
	2·0	62	0

(*d*) *Nature of polysaccharide synthesized by phosphorylase.* The polysaccharide synthesized by animal phosphorylase is generally glycogen, that synthesized by plant phosphorylase starch. This has been deduced by Cori *et al.* [1939] and by Hanes [1940] from the colour given with iodine, and has since been confirmed by X-ray spectrum analysis [Baer & Cori, 1941]. An exception to this rule is constituted by muscle phosphorylase, which acts on glucose-1-phosphate with formation of starch. We have been able to show that, prior to the formation of starch by muscle phosphorylase, a polysaccharide is formed which gives the yellow-pine colour with iodine typical of glycogen. This reaction cannot be demonstrated with fresh phosphorylase preparations acting at room temperature because starch formation in this case is too quick. If, however, the enzyme preparation is left overnight in the ice-box before it is allowed to act on its substrate, or if incubation is carried out at 0°, the yellow-pine colour reaction can be demonstrated after 5–10 min. of incubation. The colour becomes increasingly masked by the blue iodine reaction as synthesis proceeds further.

DISCUSSION

Good agreement has been found between the intensity of glycogen metabolism and the phosphorylase and phosphoglucomutase activity of various tissues. This finding favours the assumption that glycogen metabolism in all animal tissues follows the path described for muscle and liver by Cori *et al.* [1939]. An unexpectedly high phosphorylase activity and intense glycogen metabolism is shown by subcutaneous tissue.

In rat muscle almost no phosphorylase activity could be demonstrated immediately after birth. Muscle phosphorylase activity becomes considerable at the age of 14 days, when the muscles also begin to function normally. In brain, on the other hand, phosphorylase activity is already practically maximal at birth. Nevertheless, glycogen phosphorolysis in brain brei at this stage is very slow. The apparent discrepancy was found to be attributable to the lack of active phosphoglucomutase. When the mutase activity develops to normal, 14–20 days after birth, glycogen phosphorolysis too was found to become normal. This development of glycogen phosphorylation activity parallels the rise in O_2 consumption [Fazekas, Alexander & Himwich, 1941], and increases as the phosphatatic and glycolytic activities decline [Cohn & Kaplan, 1941; Epelbaum & Skvirskaya, 1940] with advancing age.

In poisoning with P, CCl_4 or $CHCl_3$, the glycogen metabolism of the liver is disturbed. In severe cases no glycogen at all could be found in liver. This is correlated with a marked fall in phosphorolytic capacity, apparently due to a decrease in the activity of phosphoglucomutase. The phosphorylase activity of liver in such cases is almost normal.

SUMMARY

1. A modified method for the preparation of phosphorylase by adsorption on $Al(OH)_3$ is described. By this method, phosphorylase can be demonstrated in all animal tissues.

2. The phosphorylase activity and the intensity of the glycogen metabolism of a tissue are shown to be positively correlated. The same is true also of the phosphorolytic breakdown of glycogen.

3. Subcutaneous tissue possesses a highly active phosphorylase, and metabolizes glycogen at a high rate.

4. Phosphorylase is not found in the muscles of rats less than 10 days of age. In 14-day-old rats the enzyme is already comparatively active.

5. Brain tissue from new-born rats shows 60% of the phosphorylase activity of the adult brain. Phosphoglucomutase in new-born rat brain, however, was either inactive or absent. This enzyme is the limiting factor in glycogen phosphorolysis up to 14–20 days after birth, after which time its activity approaches that found in the adult brain.

6. In intoxication by phosphorus, carbon tetrachloride and chloroform, liver phosphoglucomutase is inactivated, but phosphorylase activity is almost normal.

7. In adrenalectomized as well as in thyroidectomized rats no decrease in glycogen phosphorolysis by muscle could be demonstrated.

8. Some properties of the phosphorylase of muscle have been studied: on irradiation with ultra-violet light, or on heating to 55° for 20 min., complete destruction of the enzyme occurred. Adenylic acid diminished the inhibition caused by glucose in 2% concentration. With 0·3% glucose no lessening of inhibition was found when adenylic acid was added. With potato phosphorylase no inhibition of glycogen synthesis by 2% glucose was found.

9. If synthesis by muscle phosphorylase is slowed down by low temperature or by ageing of the enzyme preparation, an iodine colour reaction such as is given by glycogen can be observed after 5–10 min. of synthesis.

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The Chemical Behaviour of Dehydro-*l*-ascorbic Acid *in vitro* and *in vivo*

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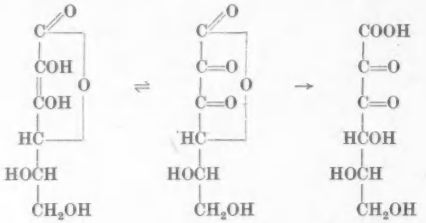
It is reasonable to assume that the function of *l*-ascorbic acid in the metabolism of the animal organism is that of a link in a co-ordinated chain of reactions. It might therefore be expected that, since ascorbic acid and dehydro-*l*-ascorbic acid constitute under certain conditions a thermodynamically reversible oxidation-reduction system, dehydroascorbic acid would become a component of these co-ordinated reactions, although under prevailing conditions it may be present in too small quantities to be detected by the available technique. With this hypothesis in view one of us started some years ago an investigation on the behaviour of dehydroascorbic acid in the guinea-pig. It was then found that whilst ascorbic acid could be almost completely recovered from the organs and tissues

of the guinea-pig soon after injection, this was not the case when dehydroascorbic acid was used. It was therefore assumed that when dehydroascorbic acid (II) was present in the organism in large quantities a part of it was reduced in the body to ascorbic acid (I) but considerable quantities were at the same time converted irreversibly into 2:3-diketo-*l*-gulonic acid (III).



I *l*-Ascorbic acid — II Dehydro-*l*-ascorbic acid — III 2:3-Diketo-*l*-gulonic acid

Before this could be demonstrated experimentally it was necessary to acquire further information on the subject. First, a direct method for estimating diketogulonic acid in small quantities in animal tissues had to be devised. In the second place, the chemistry of the conversion of dehydroascorbic acid had to be studied in greater detail than hitherto in order to be able to interpret correctly results obtained *in vivo*. The former has since been accomplished [Penney & Zilva, 1943]. The latter investigation is reported here. This communication is divided into three sections, one dealing with the purely chemical aspect of the problem performed *in vitro*, the second with the behaviour of dehydroascorbic acid and diketogulonic acid *in vivo*, and the third consisting of a general discussion of the results described in the first two sections.

The conversion of dehydroascorbic acid to diketogulonic acid was first postulated by Herbert, Hirst, Percival, Reynolds & Smith [1933] who observed that dehydroascorbic acid mutarotated in acid solution. For convenience we therefore often employ the expression mutarotation for the change of dehydroascorbic acid into diketogulonic acid, although the conversion was not measured by us polarimetrically.

I. EXPERIMENTS *IN VITRO*

Since this investigation pivots on the colorimetric determination of diketogulonic acid [Penney & Zilva, 1943] it was essential to elucidate as far as possible the chemical nature of the reactions involved. This estimation consists of two distinct phases, namely, the formation of the 2:4-dinitrophenylhydrazine derivative and the subsequent production of a red coloration on addition of alkali. The colour forms the ultimate criterion for the assessment of diketogulonic acid.

Solutions of dehydroascorbic and diketogulonic acids were prepared as described [Penney & Zilva, 1943]. To 250 ml. solutions containing 100 mg. of each of these compounds and of ascorbic acid, three equivalents of 2:4-dinitrophenylhydrazine in 100 ml. 2 *N* HCl were added. After 5 days at 40° the bulky red precipitates were filtered off, washed with dilute HCl and water and dried *in vacuo* over H_2SO_4. Table 1 gives the crude yields of the preparations and their melting points after recrystallization from acetone-ethanol. The mixed M.P. was

Table 1. *Crude yields and* M.P. *of 2:4-dinitrophenylhydrazine derivatives*

Derivative from	Crude yield % of theory	M.P. corrected
Ascorbic acid	80	279° decomp.
Dehydroascorbic acid	85	280° ,,
Diketogulonic acid	70	281° ,,

unchanged. These melting points agree well with those obtained by Herbert *et al.* [1933] for ascorbic and dehydroascorbic acid derivatives (282° and 280° decomp. respectively), and by Drumm, Scarborough & Stewart [1937] for the dehydroascorbic acid derivative (280°). Roe [1937], on the other hand, found that the dehydroascorbic acid derivative melted at 257–259° decomp. This worker apparently did not recrystallize his product, which might explain the lower M.P. The M.P. of the diketogulonic acid derivative obtained by us was the same as those of the ascorbic and dehydroascorbic acid derivatives.

The N content of the diketogulonic acid derivative (Weiler) was 20·8 % (calculated for osazone 21·0 %), which is also in good agreement with those obtained by the above workers for the ascorbic and dehydroascorbic acid derivatives.

Small quantities of each of the derivatives in 50 % acetone-ethanol were passed through a column of active alumina (Savory & Moore). In each case the adsorption was similar, a purple zone being obtained at the top of the column which could not be eluted with acetone, but was easily removed with acetic acid. No chromatographic separation could be obtained from a mixture of the three derivatives.

In view of the above results, little doubt remains that the same dinitrophenylhydrazine derivative is obtained from each of the three compounds. Indirect evidence further points to the fact that in this reaction, ascorbic acid is first oxidized to dehydroascorbic acid which is converted to diketogulonic acid by the opening of the lactone ring. That ascorbic acid is first oxidized to dehydroascorbic acid is shown by the fact that more than two equivalents of the dinitrophenylhydrazine are required

to obtain the maximum yield. Furthermore, as will be shown later, at the *p*H of the reaction mixture the mutarotation of dehydroascorbic acid is accelerated. All this evidence suggests that the dinitrophenylhydrazine ultimately condenses with diketogulonic acid in the case of all the three compounds, in support of which we found that when the H^+ concentration of the reacting solution was increased, the rate of the reaction as measured by the colorimetric method also increased (Table 2) as a consequence of the more rapid formation of diketogulonic acid.

Table 2. *Colour produced by dehydroascorbic acid in the presence of 0·2 N and 0·04 N HCl at 38°, recorded as reciprocal colorimeter readings*

HCl conc. *N*	Reciprocal colorimetric reading after 60	90	120	180	270 min.
0·2	0·0145	0·024	0·0325	0·0435	0·068
0·04	0·010	0·017	0·025	0·0325	0·0545

In another experiment the disappearance of dehydroascorbic acid was determined in the total absence of, and in the presence of, an excess of dinitrophenylhydrazine under the same conditions, i.e. in 0·4 *N* HCl at 38°. The solutions were withdrawn after various periods, cooled, and a slight excess of furfuraldehyde added to remove the remaining dinitrophenylhydrazine. Precipitation of this furfuraldehyde derivative was complete in 5 min. $KHCO_3$ was then added to neutralize partially the acid present and the final *p*H adjusted to 3·5 by the addition of McIlvaine's phosphate-citrate buffer. After filtration, the dehydroascorbic acid was determined in both cases by treatment with H_2S and subsequent titration with indophenol. The presence of furfuraldehyde in these experiments did not interfere with the determination of dehydroascorbic acid. The results are given in Table 3; it

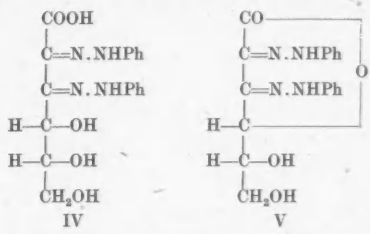
Table 3. *Recovery of dehydroascorbic acid after mutarotation in the presence and in the absence of 2:4-dinitrophenylhydrazine at 38°. HCl conc. = 0·4 N*

	Dehydroascorbic acid found (%) after 30	60	90	120	150	180 min.
Dinitrophenylhydrazine present	80	64	53	44	41	32
Dinitrophenylhydrazine absent	80	65	55	46	40	34

will be seen that the quantity of dehydroascorbic acid remaining in each case was the same within the limits of the experimental error, thus demonstrating that dinitrophenylhydrazine does not react with dehydroascorbic acid directly. The necessity for converting ascorbic acid and dehydroascorbic acid to diketogulonic acid before the reaction can take place accounts for the differential rate of the formation of the diketogulonic acid derivative, and therefore affords the possibility of determining quantitatively diketogulonic acid in the presence of the other two compounds under suitable conditions.

The evidence obtained concerning the mechanism of the formation of the deep red coloration on the addition of alkali to the dinitrophenylhydrazine derivative of diketogulonic acid was less instructive. Although the dinitrophenylhydrazine derivative of diketogulonic acid was soluble in NaOH, it was insoluble in $KHCO_3$ and Na_2CO_3. A compound or a mixture of compounds melting about 85° could be obtained from the NaOH solution by acidification. This product, unlike the original dinitrophenylhydrazine derivative, was soluble in Na_2CO_3 and $KHCO_3$ solutions. The solution of the precipitated compound turned from yellow to deep red at *p*H 9·5–10·5. It is of interest to note that the dinitrophenylhydrazone of pyruvic acid similarly changes its colour over this range, which suggests the presence of a carboxylic group in the compound.

The fact that the dinitrophenylhydrazine derivative of diketogulonic acid did not react with $KHCO_3$ or Na_2CO_3 suggests the absence of a COOH group, and from this it may be concluded that the derivative possesses either a lactone or pyrazolone structure. Ohle & Böckmann's work [1934] on the reaction of araboascorbic acid with phenylhydrazine favours the former view. They found that in the presence of HCl both the open chain (IV) and the lactone (V) forms are present and that on simple recrystallization the former readily lactonizes.



It is quite likely that under the experimental conditions prevailing in the colorimetric determination of diketogulonic acid the corresponding open-chain compound (IV) is formed, which tends to lactonize in time or on crystallization. It is therefore the open-chain compound which in all probability reacts with NaOH to form the characteristic red colour.

Mutarotation of dehydroascorbic acid under different conditions

Before investigating whether any conversion of dehydroascorbic acid takes place *in vivo* it was necessary to study in detail the mutarotation of

this substance under different conditions so as to be able to interpret the results of metabolic experiments.

Mutarotation in acid medium at 25°. It was obviously of importance to ascertain the influence of concentrations of dehydroascorbic acid on its rate of mutarotation since it could not be assumed that the kinetics of the conversion to diketogulonic acid conformed to that of a reaction of the first order. By following colorimetrically the rate of mutarotation of three concentrations of dehydroascorbic acid, i.e. 50, 200 and 1000 mg./100 ml., it was observed that the velocity of conversion (%) was increased with increase of the concentration (Fig. 1). Further experiments, however, showed that this increase was not due to the increase in concentration of the dehydroascorbic acid but to the increase in the H^+ concentration of the medium, since the dehydroascorbic acid used in these experiments was prepared by oxidation of ascorbic acid with I_2. This reaction produces HI, the concentration of which would rise as higher concentrations of ascorbic acid were employed. That the rate of mutarotation is actually accelerated by a rise in the H^+ concentration of the medium is seen from Fig. 2 which shows the effect of the addition of various concentrations of HCl. In this experiment, 15 ml. HCl, of a concentration necessary to give the final normalities recorded in Fig. 2, were introduced into each of a series of tubes kept in a water-bath at 25°. Dehydroascorbic acid solutions (100 ml.) were prepared by the oxidation of ascorbic acid (100 mg.) with I_2. The temperature of the dehydroascorbic acid solution was rapidly adjusted to 25° and 5 ml. then added to each tube. At given times, tubes were withdrawn, 2*N* $KHCO_3$ added to adjust the *p*H of the contents to 2 and the final volume made up to 50 ml. Diketogulonic acid was then determined directly by the colorimetric method, and indirectly by reduction of the residual dehydroascorbic acid with H_2S. When Cl_2 was used as the oxidizing agent instead of I_2, identical results were obtained.

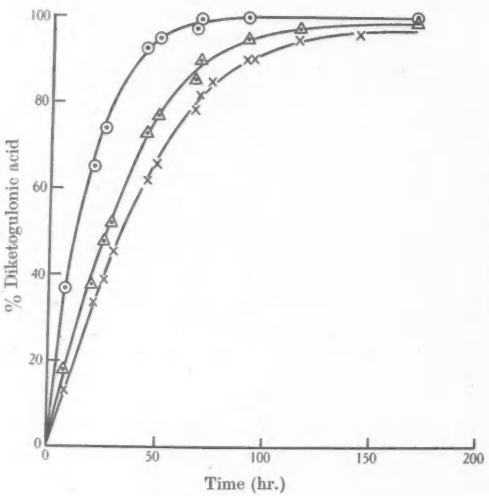


Fig. 1.

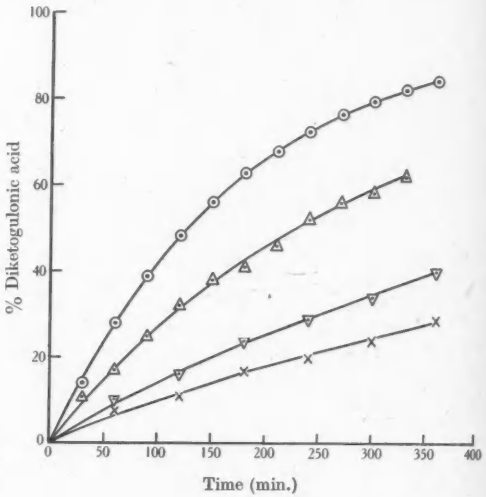


Fig. 2.

Fig. 1. Influence of initial concentration of dehydroascorbic acid on the rate of diketogulonic acid formation at 25° in the presence of HI produced during the oxidation of ascorbic acid with I_2. ×—× 50 mg./100 ml. △—△ 200 mg./100 ml. ⊙—⊙ 1 g./100 ml.

Fig. 2. Influence of hydrogen-ion concentration on the rate of mutarotation of dehydroascorbic acid at 25°. ⊙—⊙ In *N* HCl. △—△ In 0·5 *N* HCl. ▽—▽ In 0·2 *N* HCl. ×—× In 0·1 *N* HCl.

Further confirmation that the rate of conversion (%) is influenced by H^+ concentration was obtained in an experiment in which ascorbic acid was oxidized with I_2 or with KIO_3. In the former case the mutarotation was followed, with and without neutralizing the HI produced, whilst in the second case HCl equivalent to the HI produced by an equal quantity of ascorbic acid oxidized with I_2 was added to one of the neutral solutions of dehydroascorbic acid. It will be seen from Fig. 3 that the rate of conversion

(%) in each case was higher in acid solution than in neutral and was independent of the method of preparation.

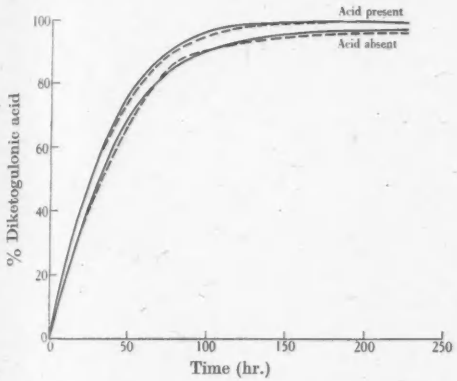


Fig. 3. —— Oxidizing agent I_2. ---- Oxidizing agent KIO_3.

The results in Table 4 show conclusively that the rate of conversion (%) of dehydroascorbic acid to diketogulonic acid is independent of concentration at a given H^+ concentration. This being so it would

Table 4. *Effect of initial concentration of dehydroascorbic acid on the mutarotation at 25°*

Initial conc. of dehydroascorbic acid mg./100 ml.	HCl conc. *N*	Diketogulonic acid (%) found after (hr.) 30	49	73
50	0·011	58	79	84
100		56	77	87
250		58	78	85
750		58	79	85
		2	3	4
25	1·0	48	63	72
100		50	66	74
200		50	65	76

be expected that the mutarotation should proceed as a reaction of the first order. By calculating all the results of this section according to the formula

$$k = \frac{2{\cdot}3 \log a/(a-x)}{t}$$

(a = initial concentration of dehydroascorbic acid; x = decrease in concentration after time t; k = velocity constant), it was found that for a given H^+ concentration the values of k were constant. The average values were: in *N* HCl, $k = 0{\cdot}0053$ min.$^{-1}$; in 0·5 *N* HCl, $k = 0{\cdot}0031$ min.$^{-1}$; in 0·2 *N* HCl, $k = 0{\cdot}0015$ min.$^{-1}$; in 0·1 *N* HCl, $k = 0{\cdot}0010$ min.$^{-1}$.

Since the conversion depends on the H^+ concentration the reaction is pseudo-unimolecular. It may also be added that so far the calibration used for the above determinations is based on the supposition that the conversion of dehydroascorbic acid to diketogulonic acid was complete. If, however, equilibrium is reached as described by Herbert *et al.* [1933] the above figures will represent the sum of the velocity constants of the direct and reverse reactions.

The action of H_2S on diketogulonic acid solutions. When the mutarotation of dehydroascorbic acid reaches completion, treatment of the resulting solution with H_2S yields an amount of ascorbic acid corresponding to about 10 % of the original content of dehydroascorbic acid. Herbert *et al.* [1933] suggested that this is due to an equilibrium between diketogulonic acid and dehydroascorbic acid. This hypothesis has been questioned by Borsook, Davenport, Jeffreys & Warner [1937] who found that by reducing a mutarotated solution of dehydroascorbic acid with glutathione at *p*H 7 reconversion of the diketogulonic acid did not take place as would have been expected in true equilibrium solution.

It was of interest to follow the mutarotation of a dehydroascorbic acid solution by determining the diketogulonic acid formed directly, by means of the colorimetric method and at the same time indirectly by difference after reducing the mutarotating solution with H_2S (on the assumption that the subsequent indophenol reduction was due to the formation of ascorbic acid from the dehydroascorbic acid of the mutarotating solution). Fig. 4 gives a graphic

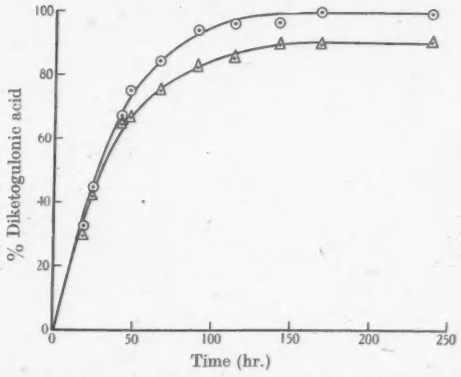
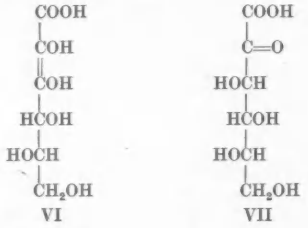


Fig. 4. The course of the mutarotation of dehydroascorbic acid at 25° as determined by the colorimetric and indirect H_2S reduction methods. ⊙——⊙ Colorimetric method. △——△ Indirect H_2S reduction method.

representation of an experiment in which the dehydroascorbic acid present (100 mg./ml.) was obtained by oxidation of a solution of ascorbic acid with I_2. The difference between the results obtained by the two methods up to about 70 % conversion falls within the limits of the experimental error.

After this a deviation eventually reaching a difference of about 10% was noted. This difference was to be expected, since, in the colorimetric determination, it was assumed that the conversion into diketogulonic acid was complete. An observation was, however, made in the course of this experiment which suggests that the change produced by H_2S in a mutarotated solution is rather more complex than might at first be expected. When diketogulonic acid was determined colorimetrically in the mutarotated solution after H_2S treatment a very low value, about 20% of that expected, was obtained. Apparently some chemical change in the diketogulonic acid had occurred on treatment with H_2S. The conversion of diketogulonic acid (III) to a tautomeric form (VI) of 2-keto-*l*-gulonic acid (VII), a compound capable of conversion into ascorbic acid [Reichstein & Grüssner, 1934], is one of the changes that can be visualized.



If this scheme be correct the presence of 10% dehydroascorbic acid in equilibrium with 90% diketogulonic acid need not therefore be postulated, and consequently the quantity of diketogulonic acid determined by the colour reaction may indeed indicate a 100% conversion. Whatever be the case the colorimetric method is applicable to the experiments to be described later, since mutarotated solutions which attained 'equilibrium' were employed in the calibration.

Influence of p*H on the mutarotation of dehydroascorbic acid at 38°.* It has already been shown that the concentration of acid in the medium influenced the rate of mutarotation. In the present experiments the mutarotation of dehydroascorbic acid was studied at higher *p*H values. The ascorbic acid was oxidized with I_2 and the solution was brought to 38° as rapidly as possible. 5 ml. dehydroascorbic acid solution were then added to each of a series of tubes containing 10 ml. McIlvaine's phosphate-citrate buffer and 20 ml. H_2O also at 38°. All buffer salts used in these experiments were recrystallized twice from glass-distilled water. For the determination of diketogulonic acid colorimetrically as well as indirectly by H_2S reduction, the tubes were removed at various intervals, cooled, and the contents transferred to a 50 ml. graduated flask, adjusted to *p*H 2 by addition of HCl, and made up to volume.

It will be seen from Fig. 5 that the rate of mutarotation as measured colorimetrically was markedly accelerated by an increase in *p*H. Whilst at *p*H 7·4 the maximum conversion of about 80% was attained in less than 15 min., only 30% conversion was recorded after 300 min. at *p*H 4. It may be

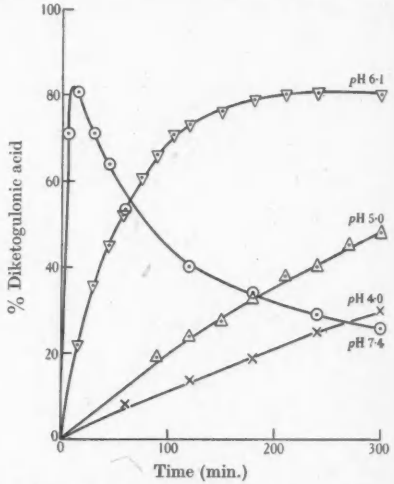


Fig. 5. The influence of *p*H on the rate of mutarotation of dehydroascorbic acid at 38°.

mentioned that at *p*H 4 or 5 the figures obtained by the two methods were almost identical, as would be expected from our previous observations (Fig. 4), since not more than 50% of the dehydroascorbic acid had been converted. It is also of interest to note that whilst at *p*H 4 and at *p*H 5 the mutarotation proceeds normally as a reaction of the first order, this is not true at *p*H 6·1. Above *p*H 6 it was found that diketogulonic acid began to decompose, the rate of disappearance increasing with increasing *p*H. The observation that maximum conversion of only 80% could be obtained at *p*H values greater than 6 could to some extent be explained by the decomposition of the diketogulonic acid. Borsook *et al.* [1937], employing electrometric methods at 35·5°, concluded that at *p*H 4·0–5·75 the mutarotation was a reaction of the first order, but at *p*H 6·43 the results obtained were too complicated for interpretation. This, as in our experiments, may have been due to the disappearance of diketogulonic acid at the latter *p*H. Ball [1937], using an electrometric technique which promoted the rapid establishment of the electrode potential, found the reaction at 30° to be of the first order between the *p*H values of 5·188 and 8·566. These results do not appear to agree completely with those obtained in our experiments and those of

Borsook *et al.*; since Ball used a technique involving rapid adjustment of electrode potential, a comparison is not strictly justified.

The discrepancy between the figures obtained by the colorimetric method and by indirect H_2S reduction at *p*H 7·4 in McIlvaine buffer is shown in Fig. 6. It will be seen that when the diketogulonic

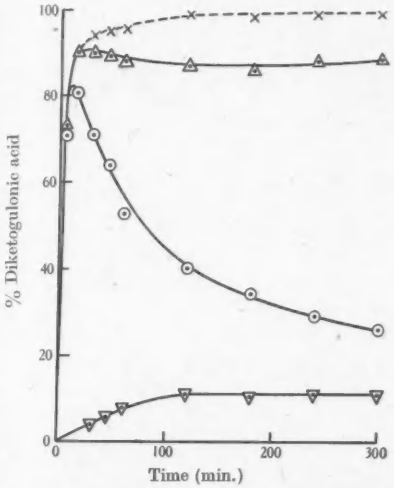


Fig. 6. The course of the mutarotation of dehydroascorbic acid at *p*H 7·4 (McIlvaine's phosphate-citrate buffer) and at 38°. ⊙——⊙ Diketogulonic acid as determined colorimetrically. △——△ Diketogulonic acid as determined indirectly by H_2S reduction. ▽——▽ Indophenol-reducing capacity before H_2S reduction. ×---× Diketogulonic acid as determined indirectly by H_2S reduction after correcting for initial indophenol-reducing capacity.

acid was determined directly a maximum value of about 80% was obtained in less than 15 min., after which time the diketogulonic acid content began to decrease, attaining a value of about 25% after 300 min. The indirect method of determining diketogulonic acid at this *p*H failed completely. Under these conditions the production of a substance which reduced indophenol without treatment with H_2S was observed. The reducing power gradually increased and eventually attained a maximum value. After treatment with H_2S the indophenol-reducing capacity was only slightly increased as will be seen from Fig. 6, in which values corrected for the initial reducing capacity are represented by an interrupted line.

That the rate of mutarotation at *p*H 4, 5 and 6 was affected by the *p*H of the solution *per se* and not by the constituents of the buffers, was indicated by the fact that identical results were obtained when phthalate buffer was used instead of McIlvaine phosphate-citrate buffer. At *p*H 7·4, when phosphate-NaOH buffer was used, the conversion of

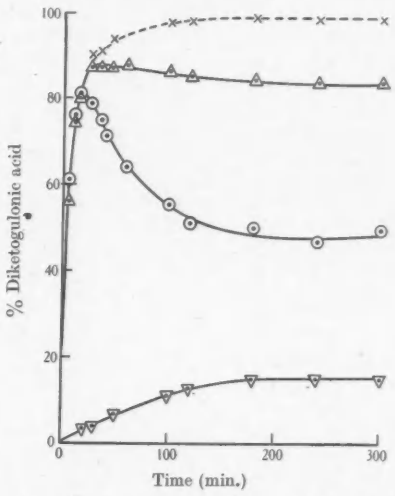


Fig. 7. The course of the mutarotation of dehydroascorbic acid at *p*H 7·4 (phosphate-NaOH buffer) and at 38°. Symbols as in Fig. 6.

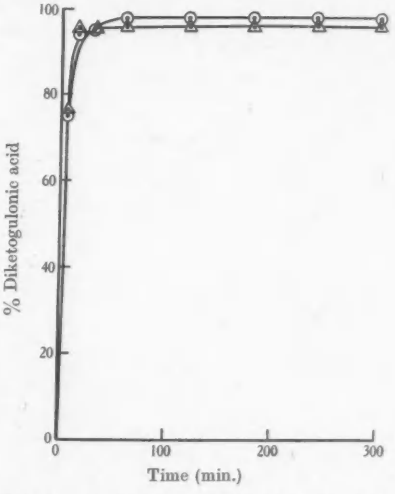


Fig. 8. Influence of borate buffer on the mutarotation of dehydroascorbic acid at *p*H 7·4 and at 38°. Symbols as in Fig. 6.

dehydroascorbic acid into diketogulonic acid and the initial decomposition of the latter proceeded as in the presence of McIlvaine buffer. After about

150 min., however, when the value recorded for diketogulonic acid was 50%, the reaction became unreliable as the colour characteristic of diketogulonic acid was no longer obtained (Fig. 7). The production of an indophenol-reducing substance was also observed when this buffer was used, the indophenol-reducing capacity again being only slightly increased after H_2S treatment. In the presence of borate buffer (*p*H 7·4), almost complete conversion to diketogulonic acid occurred, and good agreement was found between results obtained by the direct colorimetric method and the indirect H_2S reduction method (Fig. 8). The resulting diketogulonic acid was relatively stable and no indophenol-reducing substance was formed from the mutarotating solution. It is of interest to note that diketogulonic acid solution, when added to borate buffer at *p*H 7·4, did not decompose. Since borate is known to combine with polyhydroxy compounds, it might be expected to have a stabilizing effect on the diketogulonic acid.

equivalent of 2·5 mg. ascorbic acid was placed in each stopper. The tubes were then evacuated, washed with O_2-free nitrogen, re-evacuated and the contents mixed after they had attained the temperature of the bath. After various intervals the *p*H was adjusted to 2 and the diketogulonic acid determined by the colorimetric method as well as by the indirect H_2S reduction method.

A control experiment was carried out in air. The results are graphically represented in Fig. 9. It is seen at once that the course of the conversion of the dehydroascorbic acid into diketogulonic acid as measured colorimetrically was the same in both cases. Furthermore, the subsequent change in the diketogulonic acid formed in both experiments was similar to that already observed. On the other hand, the indophenol-reducing capacity of the mutarotated solution before and after H_2S treatment when carried out in the absence of air differed significantly from that observed under aerobic conditions. No indophenol-reducing substance was

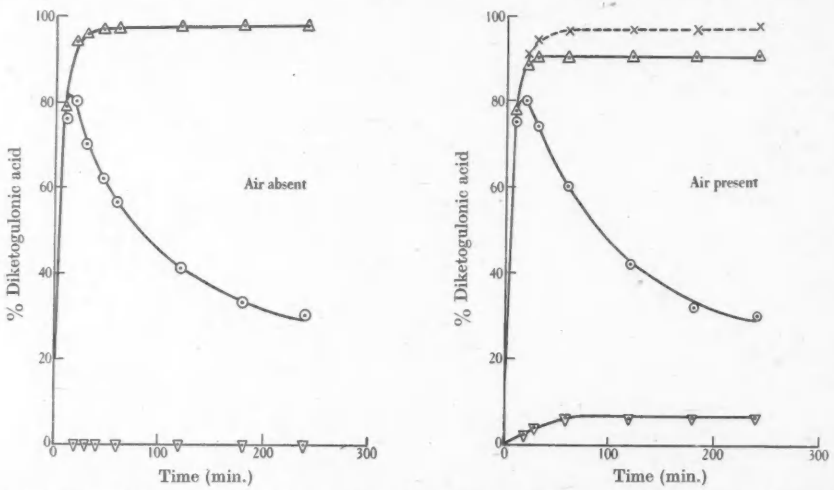


Fig. 9. The influence of O_2 on the mutarotation of dehydroascorbic acid at *p*H 7·4 (McIlvaine's phosphate-citrate buffer) and at 38°. Symbols as in Fig. 6.

Mutarotation of dehydroascorbic acid at p*H 7·4 in the presence and absence of air.* The mutarotation of dehydroascorbic acid at *p*H 7·4 in the absence of air was determined in a series of evacuated modified Thunberg tubes maintained at 38°. The dehydroascorbic acid was prepared by oxidizing a solution of ascorbic acid in oxygen-free water with I_2 and neutralizing the HI formed with NaOH. McIlvaine's buffer was also prepared in oxygen-free H_2O and was adjusted to *p*H 7·4 electrometrically. 5 ml. buffer was introduced into each of the tubes and 1 ml. dehydroascorbic acid solution containing an

produced during the course of the anaerobic experiment, and after treatment with H_2S the indophenol titration was very low. This latter reduction was of the same order as the increase found after H_2S treatment when mutarotation was allowed to proceed in the presence of air. This observation offers further evidence concerning the unreliability of determining diketogulonic acid indirectly by H_2S reduction.

Effect of temperature on the mutarotation of dehydroascorbic acid. The mutarotation of dehydroascorbic acid in the presence of HI formed by the

oxidation of ascorbic acid by I_2 (100 mg./100 ml.) was studied colorimetrically at 25 and 38°. The conversion, as would be expected, proceeded at a higher rate at 38° than at 25° (Fig. 10).

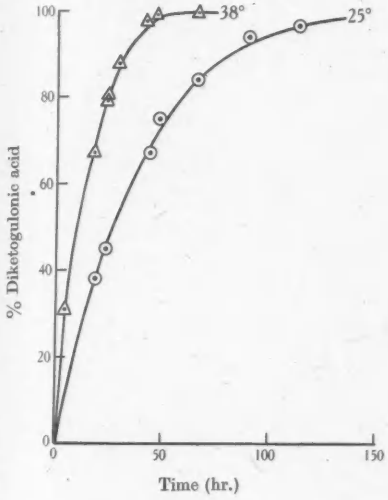


Fig. 10.

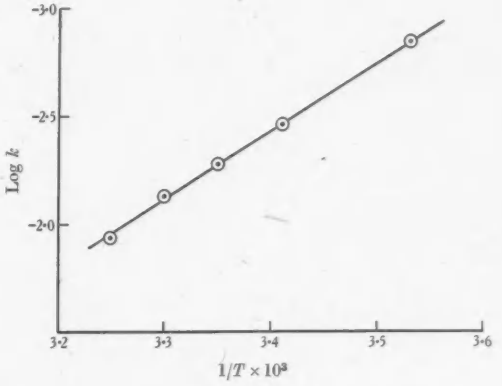


Fig. 11.

Fig. 10. Influence of temperature on the rate of mutarotation of dehydroascorbic acid in the presence of 0·011 *N* HI.

Fig. 11. Influence of temperature on the rate of mutarotation of dehydroascorbic acid in *N* HCl.

In addition, the production of diketogulonic acid was determined in *N* HCl at 10, 20, 25, 30 and 35°. The results when substituted in the formula of a reaction of the first order gave a constant for each temperature. In Fig. 11 $\log k$ is plotted against $1/T$ according to the usual integrated form of the Arrhenius equation, and it will be seen that a linear relationship exists. The temperature coefficient k_{35}/k_{25} calculated from the above data is 2·2.

II. EXPERIMENTS *IN VIVO*

The biological experiments consisted of the determination of ascorbic acid, dehydroascorbic acid and diketogulonic acid in the tissues, in the contents of the digestive tract and in the urine at various intervals after the administration of the last two compounds *per os* or parenterally to guinea-pigs weighing 300–350 g.

The dehydroascorbic acid employed in these experiments was prepared immediately before administration by the oxidation of ascorbic acid with the theoretical quantity of KIO_3. Diketogulonic acid was obtained by allowing a solution of dehydroascorbic acid, adjusted to *p*H 2 with HCl, to mutarotate for 14 days at room temperature. After this time the conversion of dehydroascorbic acid to diketogulonic acid, as assessed colorimetrically and by H_2S reduction, was complete. This solution was partially neutralized with NaOH immediately before use. Equivalents of 120 mg. ascorbic acid contained in approximately 4 ml. were used in each experiment. As the volume of the dose was high it was found convenient, when injecting, to introduce equal parts successively into each of the femoral and biceps muscles of the animals. All the guinea-pigs used in this investigation were previously kept for 5 days on a scorbutic diet to ensure depletion of ascorbic acid in their tissues, so that any formation of ascorbic acid after the administration of dehydroascorbic acid or diketogulonic acid could be detected. After administration of the dose, the animals were placed in metabolism cages for 15, 30 or 60 min., so that any urine voided could be collected for analysis. The animals were then killed after the appropriate time by stunning and bleeding, the blood being collected in oxalate. The tissues and the contents of the intestinal tract were then prepared for analysis as follows. The plasma obtained after centrifuging the blood was precipitated with an equal volume of 10% CCl_3.COOH. The contents of the stomach and intestines were obtained by ligaturing the digestive tract at the appropriate places, removing the contents by washing

out with water, making up to a given volume, and finally extracting with an equal volume of 10% CCl_3.COOH. The stomach and intestinal tissues, after drying between filter paper, and also the kidneys and liver were extracted with two parts by weight of 10% CCl_3.COOH. Urine, if present, was removed from the bladder by means of a Pasteur pipette and, if necessary, added to that passed before death. The body was then skinned and the 'carcass' was prepared for analysis as described by Zilva [1935]. After removal of the hair, the skin was minced thoroughly and a sample was extracted with two parts by weight of 10% CCl_3.COOH. Ascorbic acid and dehydroascorbic acid were then determined by titration with indophenol, the former before, and the latter after, H_2S treatment. Diketogulonic acid was assessed by a modified form of the colorimetric method already described by us [Penney & Zilva, 1943]. We found that the sensitivity of the original method could be increased by partial neutralization of the acid present in the test solution immediately after the addition of the dinitrophenylhydrazine reagent. A calibration curve was therefore constructed for this purpose by adding 0·7 ml. 2*N* $KHCO_3$ to the standard after the addition of 1 ml. dinitrophenylhydrazine. The test solutions were treated similarly, allowing for the presence of the CCl_3.COOH acid in the extracts. The standard error of percentage deviation of this modified method was 10%. This modification was not necessary in the case of urine and plasma because in these instances higher values were under consideration. The solutions obtained in the determination of diketogulonic acid in the 'carcass' were turbid; a blank reading was obtained immediately after the addition of the dinitrophenylhydrazine reagent to the extract and subsequent treatment with alkali, and used as a correction to the final reading.

The average quantities of ascorbic acid, dehydroascorbic acid and diketogulonic acid present in the tissues, stomach and intestinal contents and urine 15, 30 and 60 min. after the administration of dehydroascorbic acid or diketogulonic acid *per os* or by injection are given in Tables 5–8, together

Table 5. *Effect of injection of 120 mg. dehydroascorbic acid into the guinea-pig*

		Quantity found													
		Liver			Stomach		Small intestine		Large intestine				Excreted in		
After min.		Total mg.	mg./g.	Kidney mg.	Tissue mg.	Contents mg.	Tissue mg.	Contents mg.	Tissue mg.	Contents mg.	'Carcass' mg.	Skin mg.	urine mg.	Plasma mg./ml.	Total mg.
							Ascorbic acid								
15	Mean	11·4	0·91	0·99	0·35	0·0	2·2	0·1	1·3	0·0	12·6	4·2	0·0	0·05	33·8
	S.D.*	1·1	0·1	0·08	0·1	0·0	0·0	0·1	0·1	0·0	3·1	1·0	0·0	0·01	5·1
							Dehydroascorbic acid								
	Mean	0·1	0·01	0·05	0·01	0·0	0·0	0·0	0·0	0·2	8·9	1·7	0·2	0·0	11·1
	S.D.	0·1	0·01	0·05	0·01	0·0	0·0	0·0	0·0	0·3	2·3	1·0	0·1	0·0	3·6
							Diketogulonic acid								
	Mean	2·6	0·20	0·99	0·45	1·0	1·7	1·1	1·7	4·8	21·2	12·7	1·0	0·36	54·6
	S.D.	0·5	0·03	0·07	0·18	0·3	0·8	0·2	0·4	2·3	4·7	1·0	0·5	0·05	6·7
							Ascorbic acid								
30	Mean	14·8	1·16	1·09	0·36	0·0	2·4	0·5	1·4	1·0	9·6	3·7	0·1	0·06	35·8
	S.D.	1·1	0·07	0·35	0·06	0·0	0·3	0·1	0·1	0·9	1·7	1·1	0·1	0·02	2·3
							Dehydroascorbic acid								
	Mean	0·2	0·01	0·00	0·04	0·0	0·0	0·1	0·0	0·0	4·6	0·3	0·6	0·00	5·8
	S.D.	0·3	0·01	0 00	0·02	0·0	0·0	0·1	0·0	0·0	0·9	0·3	0·4	0·00	0·8
							Diketogulonic acid								
	Mean	3·3	0·26	1·05	0·46	0·6	1·1	0·9	1·5	5·9	17·5	10·6	3·2	0·29	50·4
	S.D.	0·5	0·06	0·16	0·11	0·2	0·3	0·4	0·3	0·8	5·6	1·0	1·6	0·01	6·2
							Ascorbic acid								
60	Mean	14·9	1·10	0·99	0·41	0·1	3·3	0·6	1·5	0·7	10·8	4·2	0·6	0·07	35·9
	S.D.	1·8	0·11	0·09	0·04	0·1	0·2	0·3	0·2	0·7	2·3	—	0·6	0·02	3·2
							Dehydroascorbic acid								
	Mean	0·0	0·00	0·00	0·02	0·0	0·0	0·1	0·0	0·7	1·4	0·6	0·8	0·00	3·0
	S.D.	0·0	0·00	0·00	0·02	0·0	0·0	0·1	0·0	0·7	2·5	—	0·5	0·00	2·2
							Diketogulonic acid								
	Mean	3·4	0·25	0·88	0·37	0·5	1·1	1·1	1·4	5·0	12·7	16·8	9·1	0·17	42·5
	S.D.	1·0	0·09	0·01	0·10	0·1	0·1	0·0	0·3	0·9	8·5	—	6·6	0·01	11·0

In this and in the following tables, it was assumed that the total volume of plasma from each guinea-pig was 15 ml.

* Standard deviation.

Table 6. *Effect of oral administration of 120 mg. dehydroascorbic acid to the guinea-pig*

After min.		Quantity found: Liver Total mg.	Liver mg./g.	Kidney mg.	Stomach Tissue mg.	Stomach Contents mg.	Small intestine Tissue mg.	Small intestine Contents mg.	Large intestine Tissue mg.	Large intestine Contents mg.	'Carcass' mg.	Skin mg.	Excreted in urine mg.	Plasma mg./ml.	Total mg.
							Ascorbic acid								
15	Mean	3·5	0·27	0·30	0·47	1·0	5·1	4·0	0·9	0·4	4·7	1·5	0·0	0·03	22·5
	S.D.	0·4	0·1	0·05	0·08	0·2	0·5	0·9	0·1	0·4	0·3	0·4	0·0	0·01	1·8
							Dehydroascorbic acid								
	Mean	0·1	0·01	0·03	0·59	51·5	0·4	3·2	0·0	0·0	1·7	0·6	0·0	0·00	58·0
	S.D.	0·1	0·01	0·03	0·03	3·9	0·3	2·6	0·0	0·0	1·7	0·4	0·0	0·00	6·5
							Diketogulonic acid								
	Mean	2·6	0·19	0·41	0·41	2·3	3·5	15·8	1·3	9·8	5·5	4·9	0·1	0·05	45·8
	S.D.	0·9	0·03	0·02	0·06	2·1	0·4	9·7	0·1	2·0	1·6	1·7	0·1	0·01	11·5
							Ascorbic acid								
30	Mean	3·5	0·30	0·45	0·48	0·9	4·7	2·8	1·1	0·5	6·1	2·7	0·1	0·05	24·1
	S.D.	0·6	0·05	0·18	0·12	0·5	0·9	1·6	0·2	0·8	2·9	1·2	0·1	0·02	5·9
							Dehydroascorbic acid								
	Mean	0·3	0·02	0·01	0·16	45·8	0·0	2·1	0·0	0·2	1·0	0·5	0·01	0·00	50·2
	S.D.	0·1	0·01	0·01	0·18	18·1	0·0	0·8	0·0	0·4	0·9	0·1	0·01	0·00	16·5
							Diketogulonic acid								
	Mean	1·9	0·17	0·37	0·40	3·3	1·9	12·2	1·2	13·1	6·6	5·9	0·7	0·05	48·4
	S.D.	0·1	0·01	0·09	0·06	0·2	0·7	3·0	0·4	10·6	1·9	2·0	0·4	0·01	9·2
							Ascorbic acid								
60	Mean	5·6	0·37	0·49	0·40	0·2	5·0	3·2	1·2	0·8	5·0	2·5	0·1	0·04	25·1
	S.D.	1·4	0·02	0·17	0·11	0·3	1·4	0·6	0·2	0·8	0·7	1·2	0·1	0·01	5·5
							Dehydroascorbic acid								
	Mean	0·2	0·01	0·01	0·06	5·0	0·2	1·2	0·0	0·0	1·0	0·6	0·0	0·00	8·2
	S.D.	0·2	0·01	0·01	0·03	1·5	0·2	0·3	0·0	0·0	0·7	0·6	0·0	0·00	2·2
							Diketogulonic acid								
	Mean	2·4	0·14	0·37	0·26	1·5	2·2	14·2	1·5	18·8	2·7	5·9	0·8	0·04	51·3
	S.D.	0·6	0·03	0·05	0·02	0·4	0·9	5·6	0·2	3·1	2·4	0·6	0·5	0·01	9·5

Table 7. *Effect of injection of 120 mg. diketogulonic acid into the guinea-pig*

After min.		Quantity found: Liver Total mg.	Liver mg./g.	Kidney mg.	Stomach Tissue mg.	Stomach Contents mg.	Small intestine Tissue mg.	Small intestine Contents mg.	Large intestine Tissue mg.	Large intestine Contents mg.	'Carcass' mg.	Skin mg.	Excreted in urine mg.	Plasma mg./ml.	Total mg.
							Ascorbic acid								
15	Mean	0·9	0·08	0·08	0·11	0·0	0·8	0·0	0·5	0·0	1·8	0·6	0·0	0·00	4·8
	S.D.	0·1	0·01	0·01	0·02	0·0	0·2	0·0	0·1	0·0	0·2	0·2	0·0	0·00	0·3
							Diketogulonic acid								
	Mean	2·6	0·20	1·19	0·48	0·7	0·9	0·9	1·3	3·8	43·4	16·0	7·5	0·55	86·6
	S.D.	0·5	0·01	0·06	0·06	0·1	0·1	0·1	0·3	0·7	0·3	1·0	1·1	0·03	2·3
							Ascorbic acid								
30	Mean	1·0	0·07	0·09	0·08	0·0	0·9	0·0	0·5	0·0	1·4	0·6	0·0	0·00	4·5
	S.D.	0·3	0·01	0·00	0·02	0·0	0·2	0·0	0·1	0·0	0·8	0·2	0·0	0·00	1·3
							Diketogulonic acid								
	Mean	3·2	0·21	1·28	0·43	0·9	1·0	0·7	1·4	4·7	26·6	15·4	11·5	0·45	73·8
	S.D.	0·7	0·01	0·12	0·08	0·6	0·2	0·2	0·3	1·6	4·3	1·0	5·5	0·01	9·6
							Ascorbic acid								
60	Mean	0·9	0·07	0·10	0·09	0·0	0·9	0·0	0·6	0·0	1·1	0·6	0·1	0·00	4·3
	S.D.	0·2	0·01	0·03	0·01	0·0	0·1	0·0	0·1	0·0	0·8	0·3	0·2	0·00	1·0
							Diketogulonic acid								
	Mean	2·3	0·17	0·90	0·35	2·1	1·1	0·6	1·2	4·6	11·7	8·9	9·3	0·23	46·5
	S.D.	0·4	0·02	0·17	0·06	0·5	0·1	0·1	0·1	0·9	8·4	2·7	7·2	0·05	18·1

No dehydroascorbic acid was detected in this experiment.

Table 8. *Effect of oral administration of 120 mg. diketogulonic acid to the guinea-pig*

		Quantity found												
		Liver			Stomach		Small intestine		Large intestine					
After min.		Total mg.	mg./g.	Kidney mg.	Tissue mg.	Contents mg.	Tissue mg.	Contents mg.	Tissue mg.	Contents mg.	'Carcass' mg.	Skin mg.	Excreted in urine mg.	Plasma mg./ml.	Total mg.
							Ascorbic acid								
15	Mean	0·6	0·05	0·07	0·12	0·0	1·0	0·1	0·4	0·0	1·6	0·5	0·0	0·00	4·1
	S.D.	0·2	0·01	0·01	0·02	0·0	0·2	0·1	0·1	0·0	0·1	—	0·0	0·00	0·3
							Diketogulonic acid								
	Mean	2·2	0·17	0·34	0·91	55·6	4·2	18·5	1·0	4·8	2·8	6·6	0·7	0·05	94·0
	S.D.	0·3	0·04	0·04	0·08	10·7	0·7	1·5	0·2	0·6	2·0	—	0·6	0·01	14·4
							Ascorbic acid								
30	Mean	0·6	0·05	0·07	0·12	0·0	1·5	0·2	0·6	0·2	0·9	0·3	0·0	0·00	4·5
	S.D.	0·2	0·01	0·03	0·01	0·0	0·2	0·2	0·1	0·3	0·8	0·2	0·0	0·00	1·8
							Diketogulonic acid								
	Mean	1·8	0·16	0·32	0·89	23·3	3·7	9·8	2·4	13·7	7·3	5·6	1·1	0·07	70·5
	S.D.	0·3	0·01	0·07	0·17	11·0	2·3	9·3	1·3	4·7	4·2	0·6	0·2	0·02	13·1
							Ascorbic acid								
60	Mean	0·7	0·06	0·08	0·13	0·0	1·4	0·6	0·7	0·5	1·5	0·5	0·0	0·00	6·1
	S.D.	0·1	0·01	0·02	0·02	0·0	0·2	0·4	0·2	0·5	0·3	0·1	0·0	0·00	0·7
							Diketogulonic acid								
	Mean	2·1	0·18	0·32	0·88	31·0	3·4	14·5	1·9	17·7	0·5	4·5	1·1	0·04	78·4
	S.D.	0·4	0·03	0·02	0·13	4·1	1·5	8·5	0·1	6·7	0·8	1·3	0·2	0·01	1·6

No dehydroascorbic acid was detected in this experiment.

Table 9. *Analysis of tissues from control guinea-pigs*

	Quantity found													
	Liver			Stomach		Small intestine		Large intestine						
	Total mg.	mg./g.	Kidney mg.	Tissue mg.	Contents mg.	Tissue mg.	Contents mg.	Tissue mg.	Contents mg.	'Carcass' mg.	Skin mg.	Excreted in urine mg.	Plasma mg./ml.	Total mg.
						Ascorbic acid								
Mean	0·6	0·05	0·04	0·10	0·0	0·6	0·0	0·5	0·0	1·4	0·6	0·0	0·00	3·5
S.D.	0·3	0·02	0·02	0·05	0·0	0·2	0·0	0·1	0·0	0·6	0·0	0·0	0·00	0·5
						Diketogulonic acid								
Mean	1·9	0·17	0·33	0·27	0·6	0·8	0·8	0·9	4·8	4·7	6·1	0·2	0·05	17·9
S.D.	0·2	0·01	0·08	0·02	0·1	0·1	0·3	0·2	1·9	1·8	0·0	0·1	0·01	6·9

No dehydroascorbic acid was detected in this experiment.

with the standard deviations. Each experimental group consists of three guinea-pigs. The concentrations of these substances in the liver are also given in the Tables. The approximate concentrations in the other tissues can be obtained from the mean weights of the organs, which were as follows: kidney 2·75 g., stomach 3·15 g., small intestine 11·0 g., large intestine 11·2 g., 'carcass' 164 g., skin 57 g. All the results are calculated as equivalents of ascorbic acid. A group of animals depleted of vitamin C for 5 days but not receiving either dehydroascorbic acid or diketogulonic acid was analysed and these results were used as controls (Table 9). All the tissues of the control animals gave a slight colour reaction, but in all probability this was due to the presence of substances other than diketogulonic acid. As the experiments were repeated several times and data of a similar trend were obtained, only representative results are given in this communication.

Significance of the biological results

Sufficient evidence has been obtained to elucidate the behaviour of dehydroascorbic acid and diketo-

gulonic acid in the living guinea-pig. When dehydroascorbic acid was introduced into the organism by intramuscular injection it was very soon partly reduced by the organism to ascorbic acid and partly converted to diketogulonic acid. The former entered the tissues. The latter was mainly excreted by the kidneys but some of it was also found deposited in some of the tissues (Table 5). As will be seen from a separate experiment, the results of which are given in Table 10, as much as about 25% of the quantity of dehydroascorbic acid injected

Table 10. *Excretion of diketogulonic acid in 24 hr. after the injection of dehydroascorbic acid or diketogulonic acid*

Day	Substance injected		Diketogulonic acid excreted in 24 hr. (mg.)		
		Animal no. ...	41	42	43
1	None		5·6	6·0	5·8
2	120 mg. dehydroascorbic acid		32·6	21·6	38·8
3	None		4·1	8·4	8·3
		Animal no. ...	44	45	46
1	None		5·8	8·7	8·2
2	120 mg. diketogulonic acid		23·5	36·8	35·1
3	None		7·6	11·7	7·2

can be excreted as diketogulonic acid in 24 hr., mostly during the first 3 or 4 hr. Similar results were obtained when diketogulonic acid was injected. Dehydroascorbic acid was estimated at the same time, and the results indicated that possibly small quantities of dehydroascorbic acid were excreted. The identity of diketogulonic acid in the urine after injection of dehydroascorbic acid was established by isolating and identifying the dinitrophenylhydrazine derivative as described previously. The crude yield was in large excess of that which would have been expected from the ascorbic acid present and was in fair agreement with the total quantity of ascorbic acid and diketogulonic acid found in the urine. M.P. after recrystallization from acetone-ethanol was 278°. Mixed M.P. with authentic derivative was 279°. N content (Weiler) = 20·7% (calculated for osazone N = 21·0%).

It will be seen from Table 5 that the concentration of ascorbic acid in the liver after injection of 120 mg. dehydroascorbic acid was very high (about 1 mg./g.). When 120 mg. ascorbic acid were injected we found that the ascorbic acid concentration was only about one-half this value. It appears that most if not all the unchanged dehydroascorbic acid which entered the blood stream and which had not mutarotated was reduced in the liver, since none was found in the blood after administration. Therefore when dehydroascorbic acid is injected the concentration of ascorbic acid in the blood is not the only factor which controls the concentration in the liver.

When dehydroascorbic acid was administered *per os* (Table 6) it passed gradually into the system but part of it remained almost entirely unchanged in the stomach before being absorbed. About 50% could be recovered as dehydroascorbic acid after 15 min. The dehydroascorbic acid in the stomach decreased with time, by absorption and passage into the small intestine, where, owing to the prevailing alkaline condition, it was converted to diketogulonic acid. No dehydroascorbic acid was found under the conditions of this experiment to reach the caecum, colon or rectum.

When diketogulonic acid was given *per os* (Table 8) it was found that most of the dose passed from the stomach along the intestine and no doubt would eventually have been excreted in the faeces either unchanged or as decomposition products. Since small quantities were found in the tissues of the digestive tract and in the urine, it may be presumed that minute quantities had passed into the blood stream although the presence of diketogulonic acid could not be demonstrated in the plasma. This may be explained by the fact that the little that entered the blood was excreted by the kidneys as fast as it passed through the intestinal tissues. Furthermore, as will be seen later, diketogulonic acid undergoes chemical change in the animal tissues. When the compound was injected the greater part was found in the 'carcass', skin and plasma (Table 7). Significant increases were also noted in the kidneys. 15 min. after injection the total amount found in the body of the animal was about 70 mg.; this quantity decreased with time and after 60 min. the amount recovered was about 30 mg., up to 15 mg. of which was found in the urine. No doubt diketogulonic acid is changed chemically in the body, as it is *in vitro* at *p*H 7·4 at 38° (cf. Fig. 9). Whether the compound was administered orally or by injection, no evidence was obtained indicating any significant lactonization of diketogulonic acid. Doubtful traces of ascorbic acid have been observed in some of the tissues and in the urine after the administration of diketogulonic acid. Their presence could be explained by the fact that mutarotated solutions were used, and these are known to yield small quantities of ascorbic acid when reduced by H_2S. Even if these minute amounts were derived from diketogulonic acid very large quantities of this open-chain compound would have to be administered in order to exert any antiscorbutic effect. It is therefore not surprising that Hirst & Zilva [1933] and Borsook *et al.* [1937] had observed no antiscorbutic effect when employing small doses of mutarotated dehydroascorbic acid.

Consideration of the total recovery of ascorbic acid and dehydroascorbic acid only after the ad-

ministration of dehydroascorbic acid shows that a great part of the dose is not accounted for. This, as can now be seen, is due to the fact that considerable quantities of dehydroascorbic acid are converted to diketogulonic acid. The *in vitro* experiments (cf. Fig. 9) explain the reason for this rapid conversion. At *p*H 7·4 and 38° the conversion of a great part of the dehydroascorbic acid into diketogulonic acid can take place within the first few minutes. As already mentioned it was observed in the preliminary experiments which led to this investigation that whilst most of the ascorbic acid could be accounted for in the body soon after injection this was not the case when dehydroascorbic acid was injected, and it was then suspected that part of the latter was converted into diketogulonic acid. This hypothesis is now fully supported by experimental evidence.

DISCUSSION

The main object of this investigation was to obtain experimental evidence which might offer some support to the hypothesis concerning the mode of action of ascorbic acid as outlined in the introduction. It was assumed that ascorbic acid was a link in a co-ordinated chain of reactions, in which it was alternately oxidized to dehydroascorbic acid and reduced to its original form. The presence of the reversibly oxidized product in minute quantities as a component available for further interaction was postulated accordingly. It is of interest now to consider how our results can be co-ordinated with this hypothesis.

Our *in vitro* and *in vivo* experiments have shown that dehydroascorbic acid tends to be converted to diketogulonic acid with comparative ease both in the presence and in the absence of O_2, the rate being influenced by the *p*H of the medium. On the above hypothesis therefore the reactions would proceed according to the following scheme:

Ascorbic acid ⇌ dehydroascorbic acid
→diketogulonic acid.

Our present evidence and that of Hirst & Zilva [1933] and of Borsook *et al.* [1937] does not favour the view that diketogulonic acid can be reconverted to ascorbic acid in the body. This is accomplished *in vitro* only by such drastic methods as treatment with HI [Herbert *et al.* 1933]. It is, however, considered that the concentration of dehydroascorbic acid must at any given time in the above reaction be extremely low, and in consequence the diminution in the quantity of the ascorbic acid concerned through the conversion of dehydroascorbic acid into diketogulonic acid would be very slow. This may account for the long time that elapses before the appearance of scurvy. In animals not capable of synthesizing the vitamin and at the same time not receiving any in the diet, the total content of the ascorbic acid of the body, after the tissues have been depleted, is very small, yet a considerable time has to elapse after the depletion before even latent scurvy can be diagnosed. The daily ascorbic acid lost during this period must therefore be so minute as to fit in with the requirements of the above hypothesis. Admittedly our results indicate no more than a bare possibility of the existence of the scheme outlined above, but they do in our opinion definitely indicate that possibility.

Another point of theoretical interest deserves mention. It was observed that when large doses of dehydroascorbic acid were given orally an increase in ascorbic acid in the tissues of the stomach was found, thus showing that dehydroascorbic acid was reduced there while passing into the blood stream. This observation offers an explanation for the economic utilization of small doses of dehydroascorbic acid. Demole [1933] and Hirst & Zilva [1933] found that dehydroascorbic acid was somewhat less active than ascorbic acid. The latter workers compared the biological response of guinea-pigs on a scorbutic diet receiving a daily oral dose of 0·25, 0·5 and 1 mg. dehydroascorbic acid with that of guinea-pigs receiving similar doses of ascorbic acid. The response of the former group was only very slightly less than that of the latter, and this was noticeable only in the two lower doses. Our present experiments suggest that doses of dehydroascorbic acid of the above order would pass quickly through the stomach wall where they would be converted into ascorbic acid. Dehydroascorbic acid in small doses would therefore begin functioning as ascorbic acid within a short time and any loss through conversion to diketogulonic acid would in consequence be very small. Large doses of dehydroascorbic acid, on the other hand, might be delayed in the stomach or in the small intestine before passing into the blood stream and would thus incur a proportionately higher loss through mutarotation.

The observations made in connexion with the parenteral introduction of dehydroascorbic acid and diketogulonic acid have, as far as we can see at present, no bearing on practical nutrition. The oral consumption of dehydroascorbic acid and even of diketogulonic acid is in all probability encountered in practice. It may occur when the tissues of vegetables, particularly those containing oxidizing enzymes, are disintegrated before being eaten. If large quantities are consumed the dehydroascorbic acid as we have seen may remain for some time in the stomach and in the small intestine before passing into the blood stream. The destruction of the antiscorbutic potency in the case of dehydroascorbic acid would be rather higher than that of ascorbic acid under similar conditions, but since this applies to quantities of vitamin C which are far greater than the physiological requirement it remains a point of theoretical interest only.

SUMMARY

(*a*) In vitro *experiments*

1. Diketogulonic acid in the presence of HCl forms a derivative with dinitrophenylhydrazine. When ascorbic acid and dehydroascorbic acid are present they are first converted to diketogulonic acid which then reacts with the reagent.

2. The percentage of dehydroascorbic acid which is converted to diketogulonic acid in a given time in the presence of mineral acids is independent of the initial concentration of the former and proceeds as a reaction of the first order at any *p*H value below about *p*H 4 investigated by us. The conversion is accelerated by increase in temperature, the temperature coefficient k_{35}/k_{25} being 2·2. At *p*H values higher or lower than about *p*H 4 the rate of conversion is accelerated.

3. Diketogulonic acid undergoes a chemical change in the presence of H_2S. At *p*H 7·4 the rate of formation of diketogulonic acid from dehydroascorbic acid, as measured by the colour reaction, is high and its decomposition proceeds at a considerable rate. This is independent of the presence of air.

The indirect determination of diketogulonic acid by H_2S treatment is not reliable under all conditions.

(*b*) In vivo *experiments*

1. When diketogulonic acid was injected intramuscularly into guinea-pigs which had previously received a scorbutic diet for 5 days, about 60% of the dose was recovered after 15 min., mainly from the 'carcass', skin and plasma. After 1 hr. only about 30%, of which as much as one-third was present in the urine, was recovered. It was evidently destroyed in the animal body.

2. When diketogulonic acid was administered orally only small quantities, if any, entered the blood. The greater part of the dose passed from the stomach along the intestine and some no doubt was excreted in the faeces.

3. When dehydroascorbic acid was injected it was partly converted into diketogulonic acid and partly reduced to ascorbic acid, some of which filled the tissues of the unsaturated guinea-pigs and some was excreted by the kidney. The liver contained abnormally high quantities of ascorbic acid. It is suggested that when large quantities of dehydroascorbic acid are introduced into the blood stream reduction takes place mainly in the liver.

4. When dehydroascorbic acid was administered *per os* it was gradually absorbed, but a considerable part of the dose remained at first (15–30 min.) unchanged in the stomach. In the small intestine, owing to alkalinity, it was converted to diketogulonic acid.

5. When 120 mg. diketogulonic acid were injected, about 25 mg. were excreted in 24 hr. The greater part of this passed through the kidney during the first 4 hr. Injected dehydroascorbic acid (120 mg.) was similarly excreted by the kidney as diketogulonic acid, in almost the same quantities.

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Experiments on the Function of Pantothenate in Bacterial Metabolism

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The purpose of this account is to record results obtained in an attempt to determine the function of pantothenate in metabolism. From the observation that pantothenate stimulated O_2-uptake and especially CO_2-output [Williams, Mosher & Rohrmann, 1936; Pratt & Williams, 1939], it was thought that this nutrient might be specifically concerned with the metabolism of α-ketonic acids, since these are an important source, perhaps the only source, of respiratory CO_2. An abstract of earlier results [Hills, 1941] showed that many other substrates may be affected. In order to determine whether this was due to an intermediary metabolite common to these substrates, experiments on interaction between substrates both aerobically and anaerobically have now been carried out, and the course of pyruvate breakdown studied. While the work was in progress an abstract of similar work, with findings differing in some details, was published by Berkman, Dorfman & Koser [1942] who concluded in a later paper [Dorfman, Berkman & Koser, 1942] that pantothenate is concerned with a metabolic step involving pyruvate or a derivative, most likely that of the conversion of pyruvate to acetate. At the same time Teague & Williams [1942] produced evidence that pantothenate was not concerned with glucose phosphorylation, glucose or hexosediphosphate fermentation, or pyruvate decarboxylation. Their test systems were pantothenate-deficient yeast extract and chick brain. Later Pilgrim, Axelrod & Elvehjem [1942] showed that diminished oxidation of pyruvate occurs in pantothenate-deficient rat liver. Although the present study has been confined to a single bacterial species, the results may therefore have wider implications.

METHODS

Organisms. On account of the simplicity of its nutritional requirements, *Proteus morganii* was chosen for this work. Since pantothenate must be supplied in the medium [Pelczar & Porter, 1940], cells deficient in pantothenate, or with sufficient for optimum metabolism, may be obtained at will by regulation of the pantothenate content of the medium. Four strains from the National Collection of Type Cultures, Nos. 1707, 2814A, 2818 and 5845, were found to have similar pantothenate requirements for growth, and a similar deficiency in O_2-uptake when grown with suboptimal amounts. The results quoted refer to strain 2818 except where otherwise stated.

Culture media. The organisms were usually grown on the 'complete' amino-acid medium described by Gladstone [1939], with the addition of 1 g. $(NH_4)_2SO_4$ and 1 g. NH_4Cl/l., and of nicotinamide (10^{-5} *M* final) and synthetic Ca (+) pantothenate (Merck), which were essential nutrients. Good crops of pantothenate-deficient organisms (afterwards described simply as 'deficient' where no ambiguity might arise) were obtained with $2–4 \times 10^{-8}$ *N* pantothenate. Aneurin (10^{-7} *M*) and riboflavin (2×10^{-6} *M*) were sometimes added to avoid partial deficiencies of these factors, but these non-essential additions appeared not to influence the results. Later it was found possible to dispense with all the amino-acids except cystine, which was probably required as a source of organic S [cf. Fildes & Richardson, 1937; Gladstone, 1937]. This NH_3-cystine basal medium was made up similarly to the NH_3 medium of Fildes [1940] with the addition of 25 mg. cystine in NaOH and 1·25 mg. nicotinamide/l. final bulk. Lactate was omitted and glucose added to the basal medium after sterilization. Pantothenate requirements were the same as for the amino-acid medium.

A basal medium consisting of 1% acid-hydrolysed casein [McIlwain, 1939] has also been used with the addition of glucose, nicotinamide and pantothenate as required. The organisms were not so deficient as those reaped from the synthetic media, even though the pantothenate concentration was reduced to 10^{-8} *M*, the minimum necessary to support adequate growth.

All media were dispensed in 100 ml. quantities in 250 ml. conical flasks. Glucose (1·25 ml. *M*) and pantothenate were added at the time of inoculation. Except where otherwise stated the amino-acid medium was used.

Inoculum. In the early stages, to avoid carrying over pantothenate, a small inoculum of *ca.* 100 organisms/ml. was used but later this proved unsatisfactory. Instead, the inoculum for 100 ml. culture was 1 ml. of an 18–24 hr. culture on the same medium with 10^{-6} *M* pantothenate, which was found to give more reproducible results than cells raked from an agar slope and suspended to give a similar opacity (*ca.* 10^8 cells/ml.). Subcultures were never made from deficient cultures, so as to avoid training the organism to dispense with an added supply of pantothenate.

The inoculated medium was incubated in air for 40–48 hr. with the small inoculum, or for 20–24 hr. with the large inoculum. The cells were collected on the centrifuge and washed once. The yield was *ca.* 10 mg. dry wt. of deficient cells/100 ml. medium, which was enough for 3–5 Warburg vessels in most experiments. Except where stated otherwise the large inoculum was used in the experiments quoted.

Measurement of metabolism. Standard manometric methods as described by Dixon [1934] were used. The cell suspension in distilled water was suitably buffered and added to a salt mixture in Warburg vessels, so that with any additions from the side-bulbs the final composition of the medium was that of Krebs & Henseleit [1932], with replacement of KH_2PO_4 by KCl, and limitation of the Ca^{++} content to $3 \times 10^{-5} N$ including the Ca pantothenate added to certain vessels. This excluded any possible effect on metabolism of the Ca^{++} in the pantothenate, and there were no difficulties due to precipitation when the $NaHCO_3$ buffer was replaced by 30 mM phosphate in the measurement of O_2-uptake by the direct method. Except where otherwise stated the pantothenate concentration was $3 \times 10^{-6} N$. In anaerobic experiments the last traces of O_2 were removed by yellow P in the centre well. The temperature was 37°.

Substrates. Pyruvate was purified by vacuum distillation according to Wendel [1932], and was stored as 5 M solution at 0–5°. It was determined by the carboxylase method according to the details of Westerkamp [1933].

Oxalacetic acid was prepared according to Wohl & Oesterlin [1901] and Wohl & Claussner [1907]. It was crystallized from hot acetone-benzene [Fenton & Jones, 1900] and determined, like acetoacetate, by the aniline citrate method [Edson, 1935; Greville, 1939]. I am indebted to Mr D. E. Hughes for pure preparations of pyruvic and oxalacetic acids. Both these substrates were neutralized just before use with the calculated quantity of $NaHCO_3$.

Except where otherwise stated the final concentration of all substrates was 224 μl. in 3·33 ml. per vessel, i.e. 3 mM (each form where *dl*-mixtures were used).

RESULTS

Pantothenate requirements for growth and metabolism

Table 1 shows the influence of different concentrations of pantothenate in the culture medium on O_2-uptake of the washed cells. The low metabolism of those grown with 1–4 $\times 10^{-8} M$ pantothenate was restored to normal by $3 \times 10^{-6} M$ pantothenate in the final suspension. If we take the yield of organisms into account, however, the amount, in deficient cells, of the enzyme requiring pantothenate was, even when metabolism had been activated subsequently by addition of pantothenate, only 55 % of that produced under optimal conditions. Table 2 shows that the concentration of pantothenate used in the final suspension was ample for optimal activation of both oxidation and fermentation of pyruvate.

Table 1. *Effect of pantothenate concentration during cultivation on O_2-uptake*

Cells grown from a small inoculum. 20 mM pyruvate added after shaking 1 hr. in air.

	Pantothenate (M) during growth		
	8×10^{-9}	4×10^{-8}	10^{-6}
Crop (mg. dry cells/100 ml. medium)	10	21	32
Q_{O_2} (μl./mg. cells):			
No pantothenate	17	16	55
With pantothenate	105	47	56
O_2-uptake (μl./100 ml. culture/hr.):			
No pantothenate	170	340	1760
With pantothenate	1050	990	1790

Table 2. *Effect of added pantothenate on aerobic and anaerobic metabolism of pyruvate by deficient cells*

Strain 2818 grown on hydrolysed casein with $2 \times 10^{-8} M$ pantothenate. The suspensions were shaken for 30 min. in the presence of the stated concentrations of pantothenate before adding pyruvate (20 mM). The data are for the period 40–70 min. after adding the substrate.

Pantothenate added M	Q_{O_2}	$Q^{N_2}_{acid+gas}$
0	54	17
10^{-9}	—	21
3×10^{-9}	59	32
10^{-8}	71	52
3×10^{-8}	81	73
10^{-7}	94	—
10^{-5}	97	73

Influence of pantothenate on oxygen uptake

Specificity of influence of pantothenate. The effect on O_2-uptake was probably specific for pantothenate, since aneurin, hydrolysed pantothenate and a partially purified preparation of cozymase were all ineffective (Table 3). The cozymase preparation stimulated the organism under appropriate conditions (cozymase-deficient cells grown on the hydrolysed casein medium with no added nicotinamide). Hence its ineffectiveness on pantothenate-deficient cells was not likely to be due to its inability to reach the active centres.

Table 3. *Specificity of influence of pantothenate on oxygen uptake of deficient cells*

Strain 2818 grown on pantothenate-deficient medium, i.e. hydrolysed casein with added pantothenate ($10^{-8} M$) and nicotinamide ($10^{-5} M$), and on nicotinamide-deficient medium, i.e. hydrolysed casein with added pantothenate ($10^{-6} M$) and no added nicotinamide.

Addition	Conc. M	Q_{O_2} (20–80 min.) Panto-thenate-deficient	Nicotin-amide-deficient
None	—	28	43
Pantothenate	$1{\cdot}5 \times 10^{-7}$	82	45
*Hydrolysed pantothenate	$1{\cdot}5 \times 10^{-6}$	34	—
Aneurin	3×10^{-5}	34	—
Cozymase	2×10^{-6}	35	80

* N HCl 10 min. at 100°.

Substrate specificity. Table 4 shows the effect of pantothenate on the O_2-uptake of deficient cells in the 1st hr. after adding a variety of substrates. The rate of O_2-uptake with pyruvate in the presence of pantothenate is taken as 100, as an arbitrary standard of comparison between different experiments. The total uptake after 4 hr. is given, expressed as mol./mol. substrate supplied. To obtain comparable results in different experiments, the cells from 200 ml. culture were always used for an experiment with 10 vessels. This procedure gave a greater uniformity than that in which a constant dry weight of organisms was used in each vessel. With the exception of glucose, for which the O_2-uptake was not greatly affected by the addition of pantothenate, and of succinate, pyruvate gave a more rapid rate of O_2-uptake than any other substrate tested.

Table 4. *Substrate specificity for influence of pantothenate on oxygen uptake of deficient cells*

To facilitate comparison of substrates with the same C skeleton but different degrees of oxidation, compounds are arranged in order of decreasing number of C atoms and increasing degree of oxidation for a given number of C atoms.

Substrate	Relative rate of O_2-uptake for 1st hr. (uptake with pyruvate and added pantothenate = 100)		Mol. O_2 Taken up in 4 hr.		Theory for completion
	No pantothenate	With pantothenate	No pantothenate	With pantothenate	
(*a*) Using organisms grown from a large inoculum					
Citrate	9	38	0·07	0·38	4·5
dl-Glutamate	17	73	0·15	0·88	4·5*
α-Ketoglutarate	14	55	0·17	0·74	4·0
Succinate	36	104	0·33	1·24	3·5
Fumarate	28	88	0·22	1·06	3·0
l(−) Malate	38	58	0·27	0·63	3·0
dl-Aspartate	20	61	0·19	0·84	3·0*
Oxalacetate	39	80	0·35	1·13	2·5
Glycerol	35	64	1·13	2·14	3·5
d- or *l*-Lactate	33	86	0·24	0·97	3·0
dl-Alanine	17	41	0·15	0·39	3·0*
Pyruvate (10 exp.)	34	100 (Q_{O_2}=75)	0·36	1·18	2·5
Standard deviation	9	(11)	0·13	0·25	—
None (11 exp.)	6	16	0·04	0·12	—
Standard deviation	3	6	0·03	0·04	—
(*b*) Using organisms grown from a small inoculum					
Glucose	150	166	1·51	2·00	6·0
Galactose	17	33	0·17	0·60	6·0
Butyrate	2	8	0·09	0·14	5·0
β-Hydroxybutyrate	8	14	0·08	0·17	4·5
Acetoacetate	11	14	0·08	0·14	4·0
Succinate	26	36	—	—	3·5
l-Malate	9	17	—	—	3·0
Propionate	4	5	0·06	0·10	3·5
dl-Glyceraldehyde	13	24	0·12	0·24	3·0*
dl-Lactate	31	74	0·37	1·18	3·0
Pyruvate (6 exp.)	39	100 (Q_{O_2}=51)	0·54	0·86	2·5
Standard deviation	6	(8)	0·10	0·13	—
Ethanol	16	22	0·23	0·39	3·0
Acetaldehyde	7	10	0·19	0·31	2·5
Acetate	8	13	0·15	0·35	2·0
Formate	33	41	0·36	0·52	0·5
None (5 exp.)	7	14	0·07	0·14	—
Standard deviation	4	6	0·03	0·04	—

* Calculated on the assumption that only one optical isomer is oxidized.

At one stage in the work it was found that malate, and to a less extent succinate, gave slower O_2-uptake (Table 4 (*b*)) than was shown by Hills [1941], the lower rate being in agreement with the report of Berkman *et al.* [1942]. Attempts to restore the activity by the addition of animal ash or Mn^{++} to the culture medium [cf. Woolley, 1941], which was suspected to be suboptimal with regard to trace elements, were unsuccessful, but with larger inocula (Table 4 (*a*)) it was found possible to get better growth and an increase in the rate of O_2-uptake in the presence of the C_4-dicarboxylic acids without any special additions to the medium. Since this study had to be abandoned before it was found possible to repeat the work on 12 out of 24 substrates using organisms grown from larger inocula, the data are not all strictly comparable and are divided between two lists: for organisms grown from (*a*) large and (*b*) small inocula respectively. As will be seen later, the latter list probably includes only two substrates, acetate and formate, which may be of importance in the function of pantothenate.

Other substrates which promoted rapid O_2-uptake (50–100 % of the rate with pyruvate) in the presence of pantothenate were: glutamate, α-ketoglutarate, glycerol and both optical isomers of lactate. Galactose, citrate, glyceraldehyde, alanine and formate gave moderate rates of O_2-uptake in the presence of pantothenate, initially 20–50 % of that with pyruvate. With formate, the rate was independent of pantothenate in the first 15 min. but fell off rapidly, more so without added pantothenate. With acetaldehyde and acetate, the initial rate was similar to that with the blank, but was maintained. Ethanol gave a slightly greater rate, while butyrate, β-hydroxybutyrate, acetoacetate and propionate, with or without pantothenate, gave rates equal to those without added substrate.

Substrate competition. As the maintenance of constant rate of O_2-uptake was more important than an attempt to determine conditions for complete oxidation, the substrate concentration in these experiments was increased to 5 m*M* (Table 5). Under these conditions, although succinate promoted rapid O_2-uptake, the effect of pantothenate was relatively small, suggesting that the previous effect (Table 4) was due to oxidation of some of the products of succinate metabolism. The smaller effect of pantothenate was not due to inability of the suspension to take up O_2 at a greater rate, since succinate and pyruvate together gave a higher O_2-uptake in the

Table 5. *Effect of other substrates on oxygen uptake of deficient cells in presence of pyruvate and pantothenate*

Exp.	Substrate, 5 m*M* each optical component	Relative rate of O_2-uptake (rate with pyruvate and added pantothenate = 100): No pantothenate	With pantothenate	With pantothenate and pyruvate
1	Pyruvate	40	100	101*
	Succinate	121	133	176
	dl-Glutamate	17	49	106
2	Pyruvate	12	100	—
	Succinate	—	—	154
	α-Ketoglutarate	—	58	119
	Oxalacetate	—	109	105
	dl-Aspartate	—	35	117
3	Pyruvate	40	100	100*
	Glucose	167	187	188

* Double pyruvate concentration.

presence of pantothenate. The bulk of the O_2-uptake with succinate therefore did not go through the same system as that with pyruvate, but the small part increased by pantothenate may have done so. With glutamate, on the other hand, the maximum rate of O_2-uptake in the presence of pyruvate was little more than that with pyruvate alone, suggesting that a common system was involved. α-Ketoglutarate and aspartate behaved somewhat similarly to glutamate, while oxalacetate, as might be expected, showed almost the same uptake with or without pyruvate. No conclusion as to the role of pyruvate in glucose oxidation can be drawn, since the failure of pyruvate to increase the uptake in the presence of glucose can have no significance in the absence of evidence to show that substrate activation was the limiting factor in the chain of oxidation.

Influence of pantothenate on anaerobic metabolism

Of the nine substrates tested, all of which gave rapid O_2-uptake, only glucose, pyruvate and oxalacetate showed anaerobic fermentation detectable manometrically in $NaHCO_3$ buffer (Table 6). The fermentation of glucose, though more rapid than that of the other substrates, was of little interest from the point of view of the function of pantothenate, since it was little influenced by this substance. A slight decrease in the rate of gas production with glucose was observed in the presence of pantothenate (presumably due to diversion of pyruvate from the oxido-reduction with triosephosphate in the Embden-Meyerhof-Parnas scheme). The reaction was complete after the evolution of nearly 2 mol. gas calculated as CO_2. The rate was uninfluenced by excess fumarate except for the continued slow evolution of gas in the presence of pantothenate after the process without fumarate had ceased. The anaerobic metabolism of pyruvate, which was already known to be markedly dependent on pantothenate (Table 2) gave about 0·75 mol. gas (0·66–0·86 in 12 experiments) calculated as CO_2,

Table 6. *Effect of added pantothenate on anaerobic metabolism of deficient cells*

Relative production of acid + gas (calculated as CO_2) from various substrates in 1st hr.: pyruvate with added pantothenate = 100. In parentheses is given mol. gas produced/mol. substrate at the time of the completion of the reaction in the presence of pantothenate. Fumarate, where present, *ca.* 3700 μl., i.e. 50 m*M*.

Substrate	With substrate alone: No pantothenate	With substrate alone: With pantothenate	With substrate + fumarate: No pantothenate	With substrate + fumarate: With pantothenate	With substrate + pyruvate: No pantothenate	With substrate + pyruvate: With pantothenate
None	0	−8	8	8	15 (0·13)	100 (0·76)
Pyruvate	15 (0·13)	100 (0·76)	50 (0·44)	286 (0·95)	—	—
Glucose	900 (1·90)	820 (1·97)	935 (1·88)	910 (2·20)	—	—
dl-Glutamate	−18	−19	—	—	25 (0·11)	154 (0·76)
α-Ketoglutarate	0	6	19	36	73	145
Succinate	−2	−20	—	—	43 (0·20)	152 (0·74)
l(−) Malate	−3	−3	—	—	64 (0·29)	300 (0·81)
dl-Aspartate	3	1	−4	12	118 (0·75)	267 (0·84)
Oxalacetate	14	59	29	73	—	—
Glycerol	4	4	17	26	—	156

suggesting that two reactions already recognized in bacterial fermentation may be taking place:

(1) $2CH_3.CO.COO^- + H_2O = CH_3.CHOH.COO^- + CH_3.COO^- + CO_2$

as in the cocci [Krebs, 1937*a*] and *Esch. coli* at an acid *p*H [Krebs, 1937*b*];

(2) $CH_3.CO.COO^- + HCO_3^- = CH_3.COO^- + HCOO^- + CO_2$

as in aerobically grown *Esch. coli* [Krebs, 1937*b*] and in streptococci [Barron & Lyman, 1939].

In the presence of excess fumarate the rate was increased nearly threefold and the yield of gas became 1 mol./mol. pyruvate. This may be explained by assuming that fumarate replaced that part of the pyruvate acting as H-acceptor as in the oxido-reduction of reaction (1). Oxalacetate gave initial rates which were less than with pyruvate, and which fell off so that it was impossible to observe the completion of the reaction. The acceleration produced by fumarate in this case was also small. α-Ketoglutarate and glycerol stimulated no evolution of gas when present as sole substrates, but gave a small amount in the presence of fumarate. Glutamate, succinate, fumarate, malate and aspartate showed no evolution of gas, while aspartate did not produce gas even in the presence of fumarate. All these substances increased the rate of gas production in the presence of pyruvate by at least 50%—in the case of the C_4-dicarboxylic acids (excepting succinate) by 100–200%—without significantly altering the total amount of gas produced. Oxalacetate gave a similar increase in rate with pyruvate which was studied in more detail (Table 7). The reaction with 112 μl. pyruvate was complete in 2 hr. when 75 μl. (0·69 mol.) gas had been evolved in excess of the blank. With 224 μl. pyruvate the rate was little increased and the reaction was not quite complete in 4 hr., but with 112 μl. pyruvate and 112 μl. oxalacetate the reaction was complete in 1 hr., the initial rate having been doubled. The yield was 1 mol. gas/mol. pyruvate. Even in the absence of pantothenate this reaction was complete in 3 hr. Exp. 2 (Table 7) showed that 0·25 mol. oxalacetate was sufficient to double the rate of fermentation, while a small effect was shown with 0·05 mol. Exp. 3 (Table 7) showed that the effect was truly catalytic, since even if the blank values were deducted (and this is not always justifiable [Krebs, 1937*b*]), the increase in output of gas due to 22 μl. (0·10 mol.) oxalacetate was 26 and 37 μl. in 1 and 2 hr. respectively, while the oxalacetate alone produced less than half these amounts. Although oxalacetate increased the rate of fermentation of pyruvate even in the absence of added pantothenate, the effect was not so great, and was possibly due to residual pantothenate in the cells. In contrast, the effects of aneurin and oxalacetate on pyruvate dismutation by aneurin-deficient staphylococci were identical either singly or together [Smyth, 1940]. Smyth suggested that the function of aneurin was the carboxylation of pyruvate to form oxalacetate, the latter acting as a catalyst in the dismutation (cf. Krebs & Eggleston [1940] for pigeon liver). In the present work, Exp. 1 (Table 7) did suggest, in fact, that an equimolecular amount of oxalacetate excluded pyruvate as a H-acceptor (thus increasing the yield of gas to 1 mol./mol. pyruvate) and that this reaction was catalysed by pantothenate.

Table 7. *Catalysis of anaerobic metabolism in the presence of pyruvate by oxalacetate with or without added pantothenate*

	Substrates		μl. acid + gas produced in periods					
			0–1 hr.		0–2 hr.		0–3 hr.	
Exp.	Pyruvate μl.	Oxalacetate μl.	No panto-thenate	With panto-thenate	No panto-thenate	With panto-thenate	No panto-thenate	With panto-thenate
1	0	0	−11	−11	−11	−10	−7	−5
	112	0	1	44	12	65	19	72
	224	0	7	51	16	100	26	134
	0	112	16	11	27	22	33	26
	112	112	41	100	70	102	104	105
2	0	0	10	6	15	10	19	10
	224	0	15	34	26	66	35	99
	213	11	20	38	31	76	42	115
	168	56	25	60	42	108	57	145
3	0	0	9	2	14	6	20	12
	202	0	24	47	40	105	56	147
	0	22	13	12	18	15	23	21
	202	22	41	83	64	151	77	165

Aerobic and anaerobic metabolism of pyruvate and the influence of pantothenate

Optimum p*H*. Table 8 shows that the optimum *p*H for pyruvate oxidation, with or without pantothenate, was about 5·9, but the optimum effect of

Table 8. *Optimum* p*H for influence of pantothenate on O_2-uptake with pyruvate as substrate*

(Initial *p*H calculated from composition of Na phosphate buffers freshly prepared in CO_2-free water.)

	Q_{O_2} 30–75 min.	
Initial *p*H	No pantothenate	With pantothenate
ca. 4·6	31	39
5·9	37	66
6·6	28	61
7·0	20	58
7·3	16	47
7·8	11	40

pantothenate occurred at about *p*H 7. Above this *p*H the proportion of O_2-uptake due to added pantothenate was almost constant at 70% of the total, suggesting that catalysis by pantothenate was being limited by the rate of formation of some intermediate, e.g. perhaps oxalacetate. Attempts to determine the optimum *p*H for fermentation by variation of the concentration of $NaHCO_3$ (Table 9)

Table 9. *Optimum* p*H for influence of pantothenate on anaerobic metabolism of pyruvate*

(Initial *p*H calculated from composition of $NaHCO_3$ buffer in equilibrium with 5·7% CO_2 in N_2.)

		μl. acid+gas			
		0–45 min. 1·5 m*M* pyruvate in *ca.* 150 m*M* salts		60–120 min. 3 m*M* pyruvate in *ca.* 280 m*M* salts	
$NaHCO_3$ m*M*	Initial *p*H	No pantothenate	With pantothenate	No pantothenate	With pantothenate
1	*ca.* 6·0	0	27	—	—
3	*ca.* 6·5	10	24	20	26
10	7·0	14	46	14	24
30	7·5	19	52	17	36
90	8·0	17	49	20	45
270	8·5	—	—	18	38

showed that it was not less than 7·5–8·0 at the salt concentrations normally used in this work. When the $NaHCO_3$ concentration was increased to 0·27 *M* to give *p*H 8·5 in equilibrium with 5·7% CO_2 (NaCl being added to other vessels to maintain the same total salt concentration) the usual lag before fermentation reached its maximum rate was prolonged for at least ½ hr., but in the 2nd hour the optimum *p*H both for total fermentation and for the increase due to added pantothenate was 8·0. Under these conditions almost exactly 1 mol. gas was produced at the optimum, while at *p*H 8·5 little more than half as much was obtained, and at *p*H 7·0 little more than one-third. These figures must be accepted with caution, however, since cessation of fermentation was not shown to be due to exhaustion of substrate, and injury of the enzyme system at slightly unfavourable *p*H values by the high salt concentration was not excluded. At the lower salt concentration, 1 mol. gas was also obtained at *p*H 8·0 however, suggesting that under optimum conditions, reaction (2) occurred to the exclusion of the oxido-reduction (1).

Aerobic disappearance of pyruvate. Table 10 shows that without added pantothenate almost exactly 0·5 mol. O_2 was taken up during the period over

Table 10. *Relationship between pyruvate disappearance and O_2-uptake with and without added pantothenate*

(Strain 1707—grown on NH_3-cystine medium.)

	μl. reactant per vessel			
	No pantothenate		With pantothenate	
Time hr.	O_2-uptake	Pyruvate present	O_2-uptake	Pyruvate present
0	—	184	—	165
1	− 46	96	−108	9
2	− 93	3	−174	3
3	−113	4	−222	0

which the pyruvate disappeared, after which the O_2-uptake continued more slowly. With added pantothenate the rate of disappearance of pyruvate was at least doubled, the rate of O_2-uptake did not fall so much and had not fallen to zero when 1 mol. had been taken up. This experiment was carried out in phosphate buffer. With repetition of the experiment (Table 11) further information was obtained by determining the disappearance of acid

Table 11. *Relationship between pyruvate disappearance,* R.Q. *and acid disappearance, with and without added pantothenate*

(Bacteria from 300 ml. amino-acid medium in 5 vessels. 1·67 ml. suspending fluid per vessel. 20 min. at 37° to reach equilibrium with 5·7% CO_2 in air before making observations.)

	μl. reactant per vessel				
	No pantothenate		10^{-5} *M* pantothenate		
	Initially	Change after 4 hr.	Initially	Change after 2 hr.	Change after 4 hr.
Pyruvate	481	−465	431	−418	−423
Bound CO_2	813	+ 75	832	+152	+216
Total CO_2	832	+572	879	+641	+769
O_2	—	−307	—	−397	−525
R.Q.		1·86		1·61	1·00

+ and − signs indicate appearance and disappearance respectively.

and formation of CO_2 by the method of Dickens & Šimer [1931] with $NaHCO_3$ as buffer. Without added pantothenate the average R.Q. over a 4 hr. period, during which all the pyruvate disappeared, approached the value 2·00, i.e. that for oxidation to acetate, though some further oxidation did occur.

With pantothenate the R.Q. was lower, even in the first 2 hr. (during which all the pyruvate disappeared), while in the second 2 hr. the data correspond to complete oxidation of some of the acetate presumably formed earlier. Unfortunately, the only evidence for the effect of pantothenate on pyruvate disappearance was that a smaller amount remained at the end of equilibration.

DISCUSSION

The evidence produced shows that the effect of pantothenate on metabolism was not confined to the oxidation of pyruvate to acetate, since a definite, though smaller, effect on O_2-uptake was seen after no measurable amount of pyruvate remained (Table 10), and possible degradation products of pyruvate such as acetate or formate themselves gave an effect on O_2-uptake (Table 4). Dorfman *et al.* [1942] concluded that the action of pantothenate may not be confined to that on pyruvate oxidation, though the bulk of their evidence was in favour of the idea that pantothenate acts on the conversion of pyruvate to acetate. It is a matter for future work to decide if the effect on O_2-uptake in the presence of acetate or formate is indirect. From this point of view the study of formate oxidation might be especially interesting since it promoted moderate O_2-uptake which was initially independent of pantothenate. In this connexion the experiments of Krebs [1937*b*] on the interaction of formate and fumarate in *Esch. coli* might be relevant. An indirect action, linked with the pyruvate system, did seem likely with the C_4 and C_5 dicarboxylic acids, on account of their effects both on O_2-uptake in the presence of pyruvate (Table 5) and on the fermentation of pyruvate (Table 6).

Whatever the role of pyruvate, however, in the oxidation of other substrates, quantitatively it was certainly the most important substrate influenced by pantothenate. Other vitamins are known to be concerned in pyruvate metabolism: (*a*) *Aneurin* as its pyrophosphate is concerned ultimately in pyruvate breakdown, though so far there is no agreement as to its precise function [cf. Barron, Lipton & Goldinger, 1941; Krebs & Eggleston, 1940; Lipmann, 1941; Quastel & Webley, 1941; Smyth, 1940]. (*b*) *Nicotinic acid* as diphosphopyridine nucleotide is concerned in the equilibrium between pyruvate and lactate, and also with that between oxalacetate and malate which, according to the views of Krebs & Eggleston [1940] and Smyth [1940], may be involved in pyruvate metabolism. (*c*) *Riboflavin* as riboflavin-adenine dinucleotide may be concerned in the later stages of H-transport, as in *Lactobacillus delbrückii* [Lipmann, 1939], though not directly concerned in substrate activation. Pantothenate should now be added to these as a component of pyruvate oxidase, though it has not been shown to play a part in the cell-free preparations from *Lactobacillus delbrückii* [Lipmann, 1939] and *Esch. coli* [Still, 1941]. Its participation, however, was not excluded since it was not shown that the preparations were free from pantothenate, and it is likely that the treatment was not drastic enough to release this factor from any protein with which it may have been bound. Pantothenate is known to be bound in liver, one of the richest natural sources, and is freed by autolysis [Williams, 1941; Williams, Truesdail, Weinstock, Rohrmann, Lyman & McBurney, 1938], but there was no autolytic process in the preparation of the enzyme of Lipmann or that of Still.

SUMMARY

1. In washed suspensions of *Proteus morganii* grown with suboptimal supplies of pantothenate, the addition of pantothenate increased the aerobic metabolism of pyruvate to a greater extent than that of 15 other substrates and the anaerobic metabolism of pyruvate more than that of nine other substrates tested.

2. The substrates which gave increases in O_2-uptake approaching that with pyruvate were six C_4 and C_5 dicarboxylic acids, and lactate. Little or no fermentation of these substrates, or of malate or glycerol, was detected manometrically, but all (except lactate, which was not tested) were shown to increase the rate of fermentation of pyruvate. With the C_4 dicarboxylic acids, including malate but not succinate, the increase was several-fold. With glutamate, α-ketoglutarate, aspartate or oxalacetate (and possibly succinate) as substrate, the increase in the O_2-uptake promoted by pantothenate tended to occur at the expense of that similarly promoted with pyruvate as substrate.

3. With glucose as substrate, fermentation was practically unaffected by pantothenate, and O_2-uptake was increased only in the later stages.

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Micro-method for Estimating Vitamin A by the Carr-Price Reaction

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In the micro-determination here described an attempt has been made to eliminate errors due to fading, differences in the colour of test and standard, and interference by non-specific absorption. The tube containing the reaction mixture is photographed in light of a selected wave-length range together with a series of standard tubes of equal dimensions (see Fig. 1), and the photographic film is analysed by means of photoelectric photometry. The advantages of this method are: (1) the working range is between 0·06 and 0·4 i.u. vitamin A in a sample; such small amounts cannot be determined by photoelectric colorimetry in the usual way, but it is necessary to work in this range when making serial investigations on the blood of small animals and is convenient in investigations on human beings; (2) rapid mixing is ensured as a consequence of the small volume of the reacting liquids (0·02 ml.); (3) a record of the colour produced can be obtained as early as 2 sec. after the initiation of the reaction, and its course can be followed photographically with accurate timing, thus avoiding the lag of galvanometer readings in photoelectric methods [Dann & Evelyn, 1938; Yudkin, 1941]; (4) the readings are independent of fluctuations in the light source during the reaction.

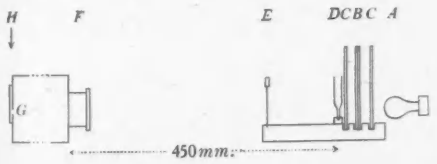

Fig. 1. Photographic arrangement. *A*, light source, two 100 W. coiled coil bulbs. *B*, space for filters: (1) 'Calorex' 2 mm.; (2) 'Signal green' 1 mm. (manuf. by Messrs Chance, 10 Princes St, S.W. 1). *C*, diffusing glass 2 mm. *D*, reaction tube and standard tubes. *E*, Wratten 26 red gelatin filter. *F*, enlarging lens at suitable distance to give image of tubes of about natural size. *G*, slit, 3·5 mm. wide, in front of slide which is moved in vertical direction. *H*, slide.

A detailed study of the error due to fading in the Carr-Price reaction is presented below. The principle of the method is generally applicable to measurement of colour in small volumes.

EXPERIMENTAL

Determination of vitamin A

Standards. Vitamin A. Any stable substance which has an absorption maximum in the region of 620 mμ is suitable as a standard. Solutions of cuprammonium sulphate in water were prepared ranging from 2·145 to 0·167 g. $CuSO_4.5H_2O$/100 ml., decreasing regularly by 20%. The solutions contained 6 ml. conc. NH_3 (sp. gr. 0·88) and 15 g. $(NH_4)_2SO_4$/g. $CuSO_4.5H_2O$. The $(NH_4)_2SO_4$ stabilizes the colour in the presence of traces of alkali from the glass. Quantities of 0·04 ml. were measured into small glass tubes measuring 2 mm. inside diameter, 2·9 mm. external diameter and 30 mm. in length, and these were then sealed. The fused end was sufficiently flat to give a negligible error in the height of the column of fluid calculated from volumes exceeding 0·01 ml. The height was measured on a mirror scale and 0·3 mm. was added for the volume of the meniscus. The average diameter of 10 tubes cut from one piece of tubing varied less than ±0·5%; the variation was ±1% in two series of tubes. In order to eliminate differences in the diameters of individual tubes correction factors were applied to the concentrations. The standards were calibrated with solutions of vitamin A-naphthoate (2–22 i.u./ml. after addition of the reagent). Original solutions in arachis oil of 1000 i.u. vitamin A-naphthoate and of 300 μg. β-carotene/g. were supplied by British Drug Houses, Ltd.

β-Carotene. Standard tubes were prepared as described for the vitamin A standards and filled with solutions of $K_2Cr_2O_7$, containing 1 ml. conc. HCl (sp. gr. 1·18)/150 mg. $K_2Cr_2O_7$, in concentrations from 0·595 to 0·01865 g./100 ml. This standard matches well with solutions of carotene and extracts of human and rat blood and rat liver in light petroleum when compared visually in daylight without light filters through the range of 1·2–36 μg. β-carotene/ml. Acid bichromate neutralizes any alkali which may dissolve from the glass and its colour is nearer to that of carotene than bichromate. Calibration was carried out with solutions of pure β-carotene in light petroleum (B.P. 40–60°). Fig. 2 shows the calibration curve as obtained by visual comparison in daylight. The smallest amount estimated was 0·004 μg. carotene in 0·003 ml.

Procedure. The reaction tube was made by joining a piece of glass tubing of a diameter equal to that of the standards to a wider glass tube of 7 mm. inside diameter, and fusing the narrow tube at such a distance as to provide a length of about 15 mm. and in such a way as to give it a flattened end. The extract freed from water was pipetted into the reaction tube and the solvent evaporated in a stream of N_2 in a bath at 50–55°. The wall of the tube was repeatedly rinsed with small amounts of light petroleum (40–60°) and the latter evaporated off. After addition of 0·005–0·03 ml. of light petroleum the tube was stoppered and centrifuged. The volume of the solution was calculated from the height of the column, allowing 0·3 mm. for the meniscus. The β-carotene layer was then mixed with the light petroleum and the carotene estimated by comparison with the bichromate standards. For many purposes no greater accuracy than ±5% is required and comparison by the eye is sufficient. Greater accuracy could be obtained by photometry, as will later be described for vitamin A.

The light petroleum was then evaporated in a stream of N_2. With extracts of blood it was sometimes necessary to transfer this solution from the small amount of precipitate present into another reaction tube with as little light petroleum as possible (0·04 ml.). The residue was taken up three times with pure $CHCl_3$, evaporated to dryness to remove all light petroleum, and finally dissolved in 0·005–0·015 ml. $CHCl_3$. Acetic anhydride was added by capillary pipette to give a final concentration of 1–1·5% and the tube kept stoppered until the $SbCl_3$ reagent was added. The acetic anhydride forming the bottom layer was then mixed with the solution and the reaction tube put in position as shown in Fig. 1. The $SbCl_3$ reagent (saturated solution in $CHCl_3$ containing 1·5% ethanol) was added from a capillary pipette having a long and very fine point. The flow of reagent was controlled by means of a piece of rubber tubing attached to the pipette. The point was kept centrally and near the surface, and the addition occupied about ½ sec. The volume of the reagent added was equal to that of the original solution, and was measured on a mm. scale in the horizontal position, 0·01 ml. corresponding to 30 mm. The first exposure of 1½ sec. was made 2 sec. after the reagent was added. Subsequent exposures of 1 sec. were made by moving the slide rapidly downwards at intervals of 1 sec. with the camera shutter open. The reaction tube was then stoppered and the volume of the liquid determined.

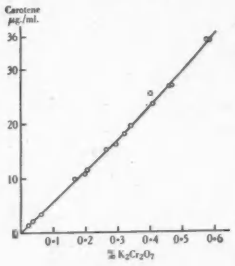

Fig. 2.

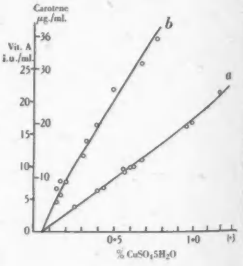

Fig. 3.

Fig. 2. Calibration curve for carotene.

Fig. 3. Calibration curve for the Carr-Price reaction of (*a*) vitamin A and (*b*) carotene.

The photographic films were analysed electrophotometrically under a microscope. A disk with a slit parallel to the photographic images to be compared was attached to the ocular mounting from which the ocular lens had been removed and a photoelectric cell of the barrier-layer

type was mounted at the upper end of the microscope. By moving the film under the microscope perpendicularly to the slit, galvanometer readings were obtained alternately for maximal transmission through the film (corresponding to maximal light absorption in a tube) and for the background of the film. The differences between the readings of the maxima and the background were plotted against the concentration of $CuSO_4$ in the standards, and the unknown value was read from the line connecting the values for the standards. This approximates to a straight line under the conditions used (Fig. 2). It is essential that the background be evenly illuminated. Photometry of three or four adjacent tubes in the range of the unknown concentration is sufficient. Fig. 3*a* shows the calibration line obtained with vitamin A-naphthoate. The arachis oil containing the vitamin A-naphthoate was diluted with light petroleum and aliquots were treated as described for extracts. The equation of the calibration line was found to be

$$y = 18{\cdot}25x - 1{\cdot}03,$$

x being the concentration of $CuSO_4.5H_2O$ as a percentage and y the concentration of vitamin A in i.u. The standard deviation was $\pm 3{\cdot}3\%$ in the range of 4–22 i.u./ml. With concentrations approaching 2 i.u./ml., corresponding to 0·04 i.u. in 0·02 ml. of reaction mixture, the error gradually rises to $\pm 15\%$.

The fading error in the Carr-Price reaction

It is generally accepted that the maximum colour of the Carr-Price reaction is proportional to the vitamin A present. Spectrographic examination confirms this view. Notevarp & Weedon [1936] showed that the blue colour obeys Beer's law. McFarlane & Sutherland [1938] achieved excellent agreement (average error 1%) between the two methods in analyses of fish-liver oil concentrates, but the error was greater with the oils themselves (average error 6%). Jensen & With [1939], analysing livers of mammals, birds and man, give the values found for $B^{1\%}_{1\,cm.}$ S 61/$E^{1\%}_{1\,cm.}$ 328 mμ as 2·70, 2·63 and 2·75 respectively, with $\sigma = 0{\cdot}404$, 0·436 and 0·406, where $B^{1\%}_{1\,cm.}$ S 61 represents the extinction coefficient of the Carr-Price reaction mixture (light filter S 61) and $E^{1\%}_{1\,cm.}$ 328 the extinction coefficient for light of wave length 328 mμ.

Variable fading rates have been observed by Dann & Evelyn [1938] not to affect the maximum colour intensity; and Meunier & Raoul [1938]

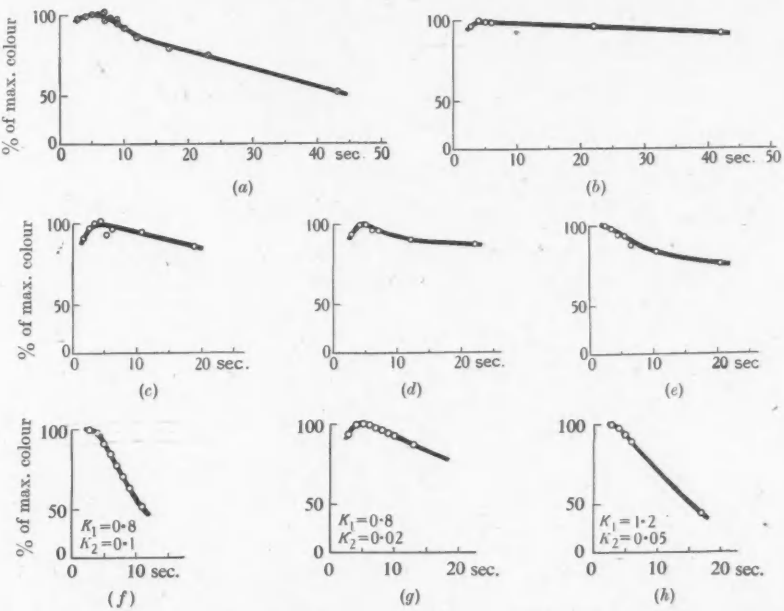

Fig. 4. Fading of Carr-Price colour given by different preparations with vitamin A activity. (*a*) Vitamin A-naphthoate: ⊘ 0·380 i.u. (16·7 i.u./ml.); ⊘ 0·283 i.u. (12·9 i.u./ml.). (*b*) β-Carotene: 0·77 μg. (35·5 μg./ml.). (*c*) Blood extract: 0·135 i.u. vitamin A (5·55 i.u./ml.); 0·143 μg. carotene (5·9 μg./ml.). (*d*) Blood extract with low concentration of vitamin A and high concentration of carotenoids: 0·054 i.u. vitamin A (2·9 i.u./ml.); 0·337 μg. carotene (16·7 μg./ml.). (*e*) Rat-liver extract: 0·264 i.u. vitamin A (13·0 i.u./ml.); 0·041 μg. carotene (2·0 μg./ml.). (*f*), (*g*) and (*h*): calculated fading curves.

suggest that they may serve as additional criteria for distinguishing vitamin A from vitamin A_2.

No information could be found concerning colour development during the first ½ min. after the start of the $SbCl_3$ reaction. This was studied by the method described above in samples of pure vitamin A-naphthoate and β-carotene, and in extracts of human blood and rat liver. If the maximum colour is reached before fading starts, these curves (Fig. 4) indicate a variable onset of the latter and variable rates of fading. If, however, fading begins as soon as the coloured compound is present, i.e. at the time of adding the reagent, the ideal maximum is never reached, but its value could be calculated by applying a correction, derived from the fading rate, to the observed maximum.

The calculations are based on a first order type reaction curve both for colour development and for fading in the neighbourhood of the maximum. The fading rate is assumed to be proportional to the concentrations of the molecules of vitamin A which had reacted with the $SbCl_3$, giving the blue colour. The relation between observed blue colour, expressed as percentage of the maximum colour assuming that no fading has taken place, and the time, is given by

$$\frac{100x}{a_0} = \frac{k_1}{k_2 - k_1}\,(e^{-k_1 t} - e^{-k_2 t}),$$

the solution of the differential equation

$$dx/dt = a_0 k_1 e^{-k_1 t} - k_2 x;$$

where x = concentration of vitamin A as chromogenic compound, a_0 = concentration of vitamin A at the beginning of the reaction, k_1 = reaction constant of the colour development, k_2 = reaction constant of the fading reaction, t = time in sec.

The values of the ordinates corresponding to a series of finite exposures as used in these experiments (1½ and 1 sec.) were calculated from

$$\frac{100}{a_0}\int_{t_1}^{t_2} \frac{x\,dt}{t_2 - t_1} = \frac{1}{k_2 - k_1}\left(-\frac{1}{k_1}\,e^{-k_1 t} + \frac{1}{k_2}\,e^{-k_2 t}\right)_{t_1}^{t_2}.$$

Fig. 4 shows curves (f, g and h) calculated by means of this formula with varying values for k_1 and k_2, in which the ordinates represent the values for $100x/a_0$ expressed as percentage of the highest value for $100x/a_0$ obtained.

It is possible to estimate k_2 directly from the slopes of the experimental curves at their points of inflexion after the maximum, when colour formation has practically stopped. From this figure and the value of the ordinate obtained at the first reading, k_1 can be determined by the formula, and with it, theoretically, the time of the true maximum.

k_2 was $0{\cdot}018 \pm 0{\cdot}003$ for vitamin A-naphthoate and 0·005 for carotene. Wide variation from 0·003 to 0·1 was observed with extracts from various sources. The value of the first ordinate in 55 experiments ranged from 90 to 100% of the maximum value found. The accuracy of the determination of k_1 was limited by the practical difficulty of ensuring complete mixing within 2 sec.

When k_1 is taken as 0·7 and k_2 as 0·01, the first ordinate works out at 88·5% of the maximum. Where k_1 is 0·7, or greater, and k_2 is 0·01, or greater, the position of the maximum according to the above formula must be at or earlier than 6 sec. after reaction. The positions of all maxima were found to fulfil this condition.

In dilutions below 4 i.u. vitamin A/ml. the curves often deviate from the calculated, the fading constant being greater at the beginning than would correspond to the value found at 22 sec. after reaction. The effect was not detected with concentrations above 4 i.u./ml. or with solutions of vitamin A-naphthoate. It may be caused by interfering substances in the blood. Agreement is obtained between curves calculated with $k_1 = 0{\cdot}8$ and experimental curves for concentrations greater than 4 i.u./ml., but the validity of the formula derived needs further test on account of the uncertainty of k_1.

Table 1. *The estimation of vitamin A and carotenoids and the fading rate of the Carr-Price colour with two concentrations*

Subject	Vol. of reaction mixture ml.	Conc. in reaction mixture i.u. vit. A/ml.	Conc. in blood per 100 ml. serum or plasma: i.u. vit. A	Conc. in blood per 100 ml. serum or plasma: μg. carotene	Serum or plasma used ml.	% fading for vit. A at 22 sec. after reaction
I.V.	0·0199	2·1	51	57	0·081	45
	0·0213	3·6	49	55	0·156	33
M.	0·0184	1·24	25	32	0·09	—
	0·0195	2·59	28	31	0·18	—
B.	0·0177	2·67	53	64	0·09	—
	0·0189	4·50	47	73	0·18	—
A.B.	0·019	4·37	103	90	0·081	24·3
	0·0213	7·52	103	90	0·156	18·2
J.R.M.	0·0187	5·27	97	78	0·102	5·5
	0·0216	7·24	87	83	0·18	8
R.B.	0·021	2·92	76	97	0·081	43
	0·0195	6·3	78	95	0·156	32
D.M.	0·0187	6·52	135	87	0·09	7
	0·0186	12·7	131	89	0·18	—
J.R.M.	0·021	4·12	85	90	0·102	—
	0·0196	7·55	82	89	0·18	—
W.	0·0177	2·66	52	74	0·09	11·7
	0·021	3·64	43	78	0·18	14·5

The observation that the percentage fading measured by the value at 22 sec. in two different concentrations of an individual extract is nearly the same (Table 1), may be taken as evidence of a first order type of fading curve. Whether the mechanism of the fading reaction is unimolecular or whether it is bimolecular with a second constituent in excess was not investigated.

It is of practical interest to know whether corrections should be applied to the maximum colour value observed in samples that fade at different rates. Table 2 gives the calculated maximum values that described for vitamin A (Fig. 3*b*). Values for concentrations lower than 8 μg. carotene/ml. were too small to be measured accurately. For the purpose of correcting the Carr-Price values of extracts

Table 2. *Calculated corrections to maxima, reached with different values of k_1 and k_2*

k_2 ...	0·005			0·01			0·02			0·03			0·05			0·1		
k_1	Max.	Corr.	f_{22}	Max.	Corr.	f_{22}	Max.	Corr.	f_{22}	Max.	Corr.	f_{22}	Max.	Corr.	f_{22}	Max.	Corr.	f_{22}
0·8	97·2	−6	5·1	94·5	−3·5	14·0	90·9	+0·5	27·5	87·6	+4	38·7	82·9	+10	57·2	73·4	+24·5	82·7
1·0	97·7	−5	5·9	95·3	−2·5	15·1	92·3	+0·5	28·9	89·4	+4	40·4	84·5	+10	58·5	76·6	+21·5	83·9

Max. = calculated maximum value of colour as percentage of theoretical total, without fading.
Corr. = corrections to experimental maximum values as percentage.
f_{22} = fading 22 sec. after reaction as percentage of the maximum.

as percentages of the theoretical values without fading (1st columns), and the corresponding corrections for samples whose constants of fading differ from those of vitamin A-naphthoate (2nd columns). The 3rd columns give the percentages of fading 22 sec. after reaction.

The distribution of the constants of fading in 33 blood extracts is given below. These figures were calculated from the values for the blue colour due to the vitamin A, and it was assumed, though not quite correctly, that the yellow colour of the extracts was entirely due to β-carotene. Possible differences in fading rate of the blue colour formed with β-carotene with various extracts were disregarded:

k_2	<0·005	0·005–0·01	0·01–0·02	0·02–0·03	0·03–0·04	>0·04
No. of samples	2	4	11	14	2	0

The corrections for the majority of extracts from blood are within ±5% (see Table 2), and so they need not be applied for practical purposes, but with fading rates greater than $k_2 = 0{\cdot}05$ the observed maxima yield values which are appreciably too low, and corrections are necessary.

The correction for β-carotene in the Carr-Price reaction

The blue colour given by carotene with the Carr-Price reagent was measured in the same way as containing carotene the calibration line was extrapolated graphically to reach the point where the calibration line for vitamin A crosses the abscissa, and this value, 0·056% $CuSO_4.5H_2O$, was subtracted from all figures of the calibration curve for carotene. The reason why the calibration line for vitamin A does not reach the zero point may be a smaller light transmission of the control compared with that of water.

SUMMARY

1. A photographic device for the measurement of the Carr-Price reaction is described.

2. The rates of fading with different extracts were studied, and the corrections which it is necessary to apply to the values obtained were determined.

3. If the concentration of vitamin A in the blood is calculated from the observed maximum colour without correction for fading the error will generally not be greater than 5%.

I wish to thank the authorities of the Haymeads Emergency Hospital, Bishop's Stortford, and of the London Hospital for the facilities which made this work possible and the British Drug Houses, Ltd. for preparations of vitamin A and carotene.

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The Effect of Prolonged Administration of Carotene in the Form of Vegetables on the Serum Carotene and Vitamin A Levels in Man

By H. HOCH (Freedom Research Scholar), *From the Haymeads Emergency Hospital, Bishop's Stortford*

(*Received 16 March 1943*)

Few systematic investigations of the effect of prolonged administration of carotene on the blood level of carotene and vitamin A have been recorded. Kauffmann & v. Drigalski [1933] found that with continued administration of carrot juice to human subjects a maximum concentration of serum carotene (0·27 mg./100 ml.) is soon reached. Vitamin A levels were not investigated.

Wendt [1935] confirmed the occurrence of maximum values for carotene in blood in spite of continued carotene intake. The serum vitamin A, which at first rose, fell again on continued administration of carotene.

Ralli, Pariente, Brandaleone & Davidson [1936] administered various doses of 0·3% carotene in oil daily for 2–4 months to normal and to diabetic persons. With the higher doses the serum carotene rose to 0·403 and 0·680 mg./100 ml. in one normal and one diabetic subject respectively during the period of dosing, and then in the normal subject further to 0·486 mg./100 ml. during the following 7 days. There was clinical evidence of 'carotenaemia' in diabetic patients when the level had risen above 0·500 mg./100 ml. Carotenaemia was not noted in normal persons. The vitamin A levels were not investigated.

The present investigation deals with the effect of prolonged administration of different amounts of carotene in the form of vegetables on the serum carotene and vitamin A levels in healthy human subjects. The circumstance that practically all food containing preformed vitamin A is rationed favoured this experiment, since the vitamin A intake could vary only within fairly narrow limits.

EXPERIMENTAL

The serum level of carotenoids and vitamin A was established in three females and two males during periods of 14–87 days prior to the feeding period. Exp. 1 and 2 were started in March 1942 after several months of a diet comparatively poor in carotene and vitamin A. The other three experiments were done during the summer. The duration of the diets with a high carotene intake ranged from 14 to 77 days. Determinations of carotene and vitamin A in the food were not carried out, but as it seemed desirable to have figures which would reflect the dietary fluctuations in these substances, detailed records of the diets were made weekly and the total consumption of carotene and vitamin A was calculated from the data shown in Table 1. The rationed food was considered separately and the deviations from the basal spring ration (approximately 10,000 i.u./week) were plotted on the charts as fractions of the total. The higher values for the vitamin A intake are due to the greater allowances of milk and cheese during the summer. The uncertainty as to the figures for the carotene content of the vegetables is greatest with those which entered the records as 'greens'. It is indicated by the shaded areas in the diagrams, the assumption being made that the biologically available carotene content might vary between 3000 and 9000 i.u./100 g.

In Exp. 1, 2 and 4 serum was analysed which had been obtained by finger prick from fasting subjects in the morning. In Exp. 5 blood was drawn from a vein and the plasma was analysed. In

Table 1. *Relative vitamin A activity of foods*

(*a*) Vegetables

From 'The vitamin A value of carotene in vegetables' [Graves, 1942]

	i.u./100 g.
Spinach	12,000
Watercress	12,000
Broccoli leaves	12,000
Carrots	7,500
Lettuce	3,000
Tomatoes	3,000
Brussels sprouts	1,700
Peas	1,000
Cabbage	1,000
French beans	700

[Jeghers, 1937]

(*b*) Rationed foods

	i.u./week	
Butter, 2 oz.	3000	[Coward, 1938]
Margarine, 4 oz.	2000	
Milk, 3 pt.	3900	Calculated from the above figure for butter, assuming 86·85% fat in butter [Winton & Winton, 1937] and 3·75% fat in milk [Coward, 1938]
Cheese, 2 oz.	420	[Davies & Moore, 1939]
Egg, 1	400	[Cruikshank, 1941]

Exp. 3 and 5 the samples were taken 2–3 hr. after breakfast. Analyses were made once a week or oftener. Carotenoids and vitamin A were estimated colorimetrically as described by Hoch [1943]. The carotenoids were expressed as μg. β-carotene and the vitamin A as i.u./100 ml. serum or plasma. Carotene and xanthophyll were not determined separately, although in Exp. 1 and 2 considerable portions of the carotenoids in blood may have been xanthophyll, due to the ingestion of spinach.

Method of extraction. The method used by Skurnik & Suhonen [1939] for the extraction of liver was employed, with modifications which prevent the presence of a protein precipitate at the beginning of the extraction with light petroleum; a heavy precipitate may impede the extraction of carotenoids and vitamin A [Yudkin, 1941]. To 0·08 (or 0·157) ml. of serum, contained in a narrow centrifuge tube (8·5 × 95 mm.) were added first 0·012 (or 0·024) ml. 40% aqueous KOH, and then, gradually, absolute ethanol to a final concentration of 30%. The air in the tube was replaced by N_2 and the tube was stoppered with a cork, fixed tightly to the tube with a loop of string, which itself was held in its place on the tube by a piece of rubber tubing. The mixture was heated in boiling water for 5 min. The tube was centrifuged to remove condensed material from the edge of the stopper before being opened, about 2 ml. of light petroleum (B.P. 40–60°) were added and the tube was then shaken for 10 min. The light petroleum was transferred to another tube of the same size with a special pipette (Fig. 1). Pipettes of this shape proved useful for the transfer of volatile fluids, the spilling of fluid from the pipette by too strong suction being prevented by the double bend. To the alkaline serum-ethanol mixture in the first tube absolute ethanol was added to raise the concentration to 50%. The mixture was again heated, under N_2, for 1½ min. The tube was centrifuged and, after addition of about 2 ml. light petroleum, was shaken as before. The pooled extracts were washed three times with distilled H_2O, once with about $2N$ HCl* and again three times with H_2O. The layers were separated by centrifuging and the bottom layer was removed with the pipette which had been used for transferring the light petroleum. The extract was then dried over anhyd. Na_2SO_4, evaporated to dryness, taken up with light petroleum and analysed for carotenoids and vitamin A.

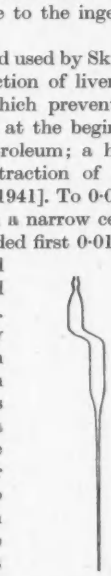

Fig. 1. Pipette for transferring volatile liquids.

Fig. 2 and 3 illustrate the results obtained in Exp. 1 and 2. The average intake of vegetable sources of carotene was ½ lb. of carrots/day or its carotene equivalent in other vegetables, mainly spinach (see Table 1), for periods of 63 and 77 days.

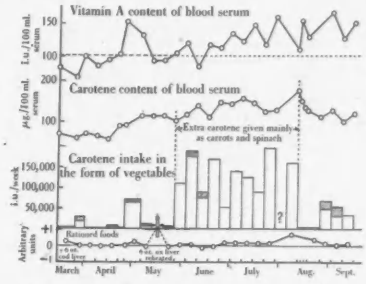

Fig. 2. Exp. 1. A.B., ♀, 24 years; ←——→ period of average weekly intake of about 120,000 i.u. of carotene, mainly as carrots and spinach.

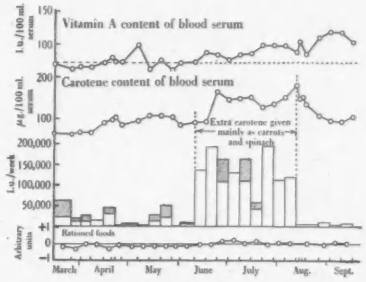

Fig. 3. Exp. 2. R.B., ♀, 24 years; ←——→ period of average weekly intake of about 120,000 i.u. of carotene, mainly as carrots and spinach.

The carotene level of the serum showed an increase from 60 to about 100 μg./100 ml. in the period before extra carotene was given, due to increased intake of vegetables in the free diet. It then increased further to about 150 μg. and this level was maintained during the period of carotene feeding with fluctuations parallel to the intake. On discontinuation of the surplus of vegetables the carotene level fell slightly (to 115 and 90 μg.) in Exp. 1 and 2. In Exp. 5 the plasma-carotene values varied between 120 and 160 μg. before and during the feeding period and between 150 and 180 μg. after the feeding period.

* In a few cases, previous to the use of HCl at this stage, turbid reactions were obtained with the $SbCl_3$ reagent in spite of the presence of acetic anhydride. With the procedure described here, the reaction mixture was always optically clear. The absence of the Tyndall effect was ascertained in every case.

The serum vitamin A level started to rise, apparently within the first 2 weeks of the feeding period, in Exp. 1 and 2. The average value changed in Exp. 1 from 102 before, to 147 i.u./100 ml. after, the feeding period, and in Exp. 2 from 71 to 105 i.u./100 ml. The vitamin A level remained unaltered in Exp. 5 at about 150 i.u./100 ml.

A different reaction was observed in Exp. 3 and 4 (Fig. 4), in which the diet was supplemented with 1 lb. of carrots daily for 25 and 32 days respectively. The carrots were included in the meals and consumed together with about 3% of margarine or butter. They were steamed or otherwise cooked, or (Exp. 4) grated, and steamed with the margarine for 30–40 min. The carotene level rose continuously till the 19th and 20th day to 375 and 470 μg./100 ml. At this time the rate of increase of the serum carotene

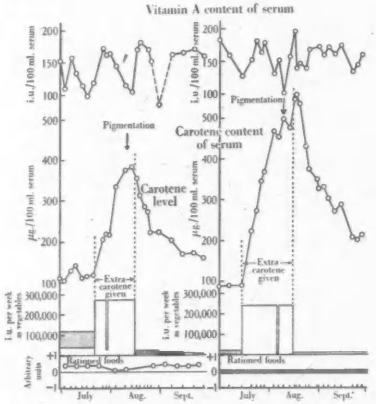

Fig. 4. Exp. 3. N.D.H., ♀, 29 years. Exp. 4. H.H., ♂, 33 years. ←——→ period of average weekly intake of about 240,000 i.u. of carotene as carrots.

slowed down considerably in spite of continued intake of carrots for further periods of 6 and 12 days respectively. The level remained high in case 4 for a further 4 days and it fell in both cases first at a rapid rate, then more slowly. The highest levels reached were 383 and 557 μg./100 ml. The vitamin A levels fluctuated around 143 and 155 i.u./100 ml. during the whole experiment.

At about the time at which the initial rate of increase in the level of carotene changed, the skin of both subjects became yellow, the pigmentation, particularly of the face and hands, being intense on the 20th and 26th day respectively. The sclera showed no discoloration. The pigmentation persisted with decreasing intensity for more than 16 days after discontinuation of the carrots in the diet.

DISCUSSION

The concentration of carotene in the serum must depend on the difference between its rate of uptake into the blood and its rate of elimination, which might include chemical destruction, excretion, and deposition in stores or in tissues where it is converted into vitamin A.

The statement that with a moderate intake of carotene the serum carotene fluctuates with the intake was confirmed in the present experiments. In two cases, however, in which there was a high intake of carrots (Fig. 2), some characteristic features of the carotene curves obtained allow the following interpretation.

The increase in serum carotene was very nearly linear during the first part of the feeding period. This steady increase makes it probable that the rate of uptake into the blood, as well as the rate of elimination, was constant. The time at which the bend of the curve occurs, after the ascending part, coincides almost exactly with the visible deposition of carotene in the skin. Assuming that this deposition, together perhaps with a deposition in other less common sites, started while the other mechanisms remained essentially unchanged, then its rate must be smaller than, or, at the most, equal to, the initial rate of increase. The total amounts thus calculated to be deposited daily in these new sites are very small compared with the amounts consumed.

The bend on the descending limb of the curve, after the almost linear fall for 10 and 8 days respectively, indicates an additional output of carotenoids from stores. The slow fading of the visible pigmentation, together with the abrupt fall of the serum carotene after the maximum, suggest a much retarded release of carotene from the skin and the stores.

It is remarkable that the levels of vitamin A in these two cases, and also in one case with medium carotene intake (Exp. 5), did not rise significantly above the levels (126, 155 and 150 i.u./100 ml.) which were found at the beginning of the experiments. A significant increase in the serum content of vitamin A on feeding medium doses of carotene was observed in two cases after a period of low vitamin A and carotene intake during the winter. The original levels were 102 and 71 i.u./100 ml., and the increase seemed to have started within the first 2 weeks. The intake before the experiment thus appears not to have been optimal. The vitamin A values were in all cases within the range considered as normal.

SUMMARY

1. The intake of carotene in the form of spinach and carrots in amounts corresponding to a biological activity of 120,000 i.u./week raised the serum

carotenoid content, expressed as β-carotene, from about 100 to 150 μg./100 ml. in two cases, and from 120 to 160 μg./100 ml. in a third case.

2. The vitamin A levels rose in two cases from 102 and 71 to 147 and 105 i.u./100 ml. respectively, and remained unchanged at about 150 i.u./100 ml. in the third case.

3. Consumption of amounts of carrots sufficient to supply about twice the biological activity provided in the first three cases, resulted in a rapid rise in the serum carotene content from 120 to 375 and 470 μg./100 ml. within 19 and 20 days respectively. Pigmentation of the skin appeared in both cases and it was intense on the 20th and 26th days.

4. The vitamin A levels did not change significantly. The single values fluctuated in the neighbourhood of 143 and 155 i.u./100 ml. during the whole experiment.

I wish to thank the authorities of the Haymeads Emergency Hospital, Bishop's Stortford, for the facilities which made this work possible.

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The Vitamin B_1 Content of National Flour and Bread—the Results of Comparative Tests by Various Methods*

A Report by
THE VITAMIN B_1 SUB-COMMITTEE OF THE ACCESSORY FOOD FACTORS COMMITTEE OF THE MEDICAL RESEARCH COUNCIL AND THE LISTER INSTITUTE

(*Received 20 April 1943*)

At the request of the Ministry of Health, an investigation has been made into the merits of the various methods available for the determination of vitamin B_1† in national flour, in order to arrive at the most suitable procedures for use in chemical laboratories where food analyses are regularly made. The present report includes a comparison of chemical with biological and microbiological assays. The vitamin B_1 content of bread made from one of the flours was also examined in order to determine the effect of baking.

The flours employed were authentic specimens of national flour, i.e. 85% extraction flour as defined in the Medical Research Council's second report on bread [Medical Research Council, 1941]. The bread was baked in a batch of 40 loaves of 2 lb. each by a usual commercial procedure. The loaves were sliced a few hours after baking and the slices placed on slatted shelves and dried at 37°. Drying was complete in 2 days when the bread, crust included, was powdered in a laboratory mill.

* The following laboratories and investigators took part in the tests organized by the Committee:

(A) F. W. F. Arnaud, County Analyst's Laboratory, Kent. (B) A. M. Copping, Lister Institute of Preventive Medicine. (C) H. E. Cox, Public Analyst. (D) E. R. Dawson, Distillers Co. Ltd., Research Laboratories. (E) L. J. Harris and Y. L. Wang, Dunn Nutritional Laboratory. (F) E. Hinks, Public Analyst. (G) E. B. Hughes, J. Lyons and Co. Ltd., Laboratories. (H) G. V. James, Roche Products Ltd., Analytical Laboratories. (I) D. W. Kent Jones, the Dover Laboratories. (J) S. K. Kon and S. Y. Thompson, National Institute for Research in Dairying. (K) T. Moran and R. G. Booth, Research Association of British Flour-Millers. (L) B. S. Platt and G. E. Glock, Nutrition Building, National Institute for Medical Research. (M) W. M. Seaber, Consulting and Agricultural Chemist. (N) W. Smith, Allen and Hanbury Ltd., Analytical Dept. (O) F. Wokes and J. G. Organ, 'Ovaltine' Research Laboratories.

† I.e. aneurin, known as thiamin in the United States of America; for the purpose of this report vitamin B_1 includes aneurin as it occurs in biological materials.

METHODS

Biological

Early work on which the biological methods are based is summarized in the Medical Research Council [1932] report: 'Vitamins: A Survey of Present Knowledge' (p. 149 for 'growth' and 'curative tests' and p. 137 for 'bradycardia' in rats). The rat-growth method (as employed by Copping) was that described by Chick & Roscoe [1929]. The rat-growth method (as employed by Harris), the rat bradycardia method and the 'cure of convulsions' method were described by Harris [1940–1].

Microbiological

The literature relating to the original observations which led up to the fermentation method is briefly reviewed in the Medical Research Council [1932] report quoted in the preceding paragraph (pp. 170–1). The method used in the present investigation is that of Schultz, Atkin & Frey [1942].

Chemical

Thiochrome method. The two procedures adopted by the Sub-Committee are given in full in Appendix A, with references to the literature.

Azo method. The method used is that described by Platt & Glock [1943] and is based on the formaldehyde azo method of Kinnersley & Peters [1927], and Prebluda & McCollum [1936].

RESULTS

The results obtained in the collaborative tests by the various methods employed are set out in Table 1. They show that all methods of estimation yield values which are in good agreement, especially for the flours.

Table 1. *Vitamin B_1 content of national flours and bread determined by various biological, microbiological and chemical methods*

(All values are given as μg./g. dry material and are averages of several estimations.)

Method	Flour C	Bread-crumb C	Flours: AGS60	N.M. 116/42	N.M. 134/42	D	E	F	Investigator (see footnote, p. 433)
Biological:									
Rat growth: ♂	4·3	4·3	—	—	—	—	—	—	E
♀	4·4	4·9	—	—	—	—	—	—	E
(i)	3·1	3·3	—	—	—	—	—	—	B
(ii)	4·9	3·3	—	—	—	—	—	—	B
Cure of convulsions	3·2	3·0	—	—	—	—	—	—	E
Bradycardia	4·2	3·2	—	—	—	—	—	—	E
Average	4·0	3·7	—	—	—	—	—	—	
Microbiological:									
Fermentation	4·1	3·2	—	3·6	3·9	—	—	—	D
Chemical. Thiochrome:									
Visual comparison	3·5	—	—	—	—	—	—	—	C
	3·8	3·0	—	—	—	—	—	—	D
	4·2	3·4	—	4·3	4·2	4·2	3·9	4·2	E
	3·3	—	—	—	—	—	—	—	F
	4·0	3·2	3·0	3·9	3·8	3·6	3·4	—	G
	3·5	2·6	3·0	3·5	3·5	—	—	—	I
	3·6	3·2	—	—	—	—	—	—	M
Average	3·7	3·1	3·0	3·9	2·8	3·9	3·7	—	
Photoelectric comparison	3·9	2·9	3·0	3·4	3·5	—	—	—	D
	3·6	2·5	2·9	3·9	3·5	—	—	—	H
	3·5	2·8	2·9	3·4	3·5	—	—	—	I
	4·1	3·7	—	3·6	3·3	—	—	—	J
	3·9	3·0	3·0	3·6	3·6	3·7	3·1	3·8	K
	3·4	2·5	3·0	3·2	3·4	—	—	—	N
	3·7	3·2	3·0	3·3	3·3	—	—	—	O
Average	3·7	2·9	3·0	3·5	3·4	—	—	—	
Chemical. Azo	4·0	—	—	—	—	—	—	—	A
	3·5	—	—	—	—	—	—	—	C
	—	—	—	—	—	4·3	3·8	—	E
	3·6	—	—	—	—	—	—	—	F
	3·9	—	3·0	3·8	—	3·2	3·0	3·5	G
	4·4	—	—	—	—	2·8	3·1	3·5	L
Average	3·9	—	—	—	—	3·4	3·3	3·5	

The results of a statistical examination of the figures given in Table 1 are shown in Tables 2 and 3. In Table 2 are the values for vitamin B_1 for flour C and for breadcrumb C obtained from flour C, as determined by the biological and chemical tests.

Table 2. *Comparison of vitamin B_1 content ($\mu g./g.$) of flour C and breadcrumb C*

	Biological tests		Chemical tests	
	As received	Calculated on dry wt.	As received	Calculated on dry wt.
Flour C	3·9	4·4	3·4	3·9
Breadcrumb C	3·6	3·9	2·8	3·0

The average figures given were obtained by weighting the various results according to their accuracy.

The accuracy of these results has been determined and is set out in Table 3.

Table 3. *The limits of error of the results given in Table 2*

	Biological tests, %		Chemical tests, %	
	$P=0·95$	$P=0·99$	$P=0·95$	$P=0·99$
Flour C	76·4–130·9	70·2–142·5	$\pm 1·71$	$\pm 2·27$
Breadcrumb C	70·8–141·2	63·5–157·5	$\pm 4·18$	$\pm 5·49$

The difference between values of flour C and breadcrumb C is covered by the error of the test in the biological determination, therefore biologically they are apparently equal in value; but the difference in the chemical test is not covered by the error of the test, therefore chemically they are apparently of unequal value. In other words, there appeared to be a loss of vitamin B_1 on baking as judged by the chemical test, but the biological test was not accurate enough to confirm this.

Only four experiments, two or three observations in each by one observer, were made by the fermentation method, and data were insufficient to make any reliable comparison of the accuracy of this method with that of the chemical methods.

The limits of error obtainable by chemical methods in routine determinations of pure vitamin B_1 and of vitamin B_1 in national flours and bread are shown in Table 4. The calculation for pure vitamin B_1 by the thiochrome visual method was based on the results of a series of 24 determinations made by Dr Y. L. Wang on one solution of vitamin B_1. That for pure vitamin B_1 by the thiochrome photoelectric method was based on duplicate determinations on 100 different solutions made by Dr R. G. Booth. The calculation for the flours and bread is based on 61 observations (five observers) for the thiochrome method by visual comparison and 80 observations (seven observers) for the same method using photoelectric comparison. There were 22 observations (two observers) for the azo method.

It is doubtful if the photoelectric method is less accurate than the visual method; it is certainly not more so. It is also doubtful if the azo method is more accurate than the thiochrome methods, as fewer workers on the former sent in results on which to base an estimation of the accuracy of this method.

CONCLUSION

The thiochrome and azo methods as used in this work are considered to be reliable for the routine determination of vitamin B_1 in national flour.

(*Signed*) R. A. Peters (*Chairman*)
H. Chick
K. H. Coward
L. J. Harris
B. S. Platt
T. F. Macrae (*Secretary*)

The Committee is indebted to the Research Association of British Flour-Millers for supplying and distributing specimens of national flour; to Messrs Matthew, Cambridge,

Table 4. *Limits of error obtainable in chemical determinations of pure vitamin B_1 and of vitamin B_1 in extracts obtained from national flours and bread*

	Thiochrome method, %				Azo method, %	
	Visual comparison		Photoelectric comparison			
	$P=0·95$	$P=0·99$	$P=0·95$	$P=0·99$	$P=0·95$	$P=0·99$
Pure vitamin B_1:*						
In duplicate	1·97	2·59	1·98	2·60	2·8	3·7
In triplicate	1·61	2·11	1·62	2·12	2·3	3·0
In quadruplicate	1·39	1·84	1·40	1·84	2·0	2·6
Flour or bread:†						
In duplicate	11·2	14·7	13·2	17·4	8·9	11·6
In triplicate	9·1	12·0	10·8	14·2	7·2	9·5
In quadruplicate	7·9	10·4	9·3	12·2	6·3	8·3

* The two sets of results obtained for the comparison of the thiochrome and the azo methods, are each the results of work done in only one laboratory. The limits of error by the azo method are for an amount of $30\mu g$. Similar values have been obtained for amounts of $20–40\mu g$.

† The errors for flours and bread include errors due to differences between laboratories.

for baking the bread; to Miss A. M. Copping for drying and distributing the bread; to Mr H. W. Kinnersley for submitting a memorandum on the technique of the thiochrome method; to Dr E. B. Hughes representing the Society of Public Analysts and Other Analytical Chemists; to Dr K. H. Coward and Dr J. C. Irwin for statistical analysis of the results; to the laboratories that carried out the trials and to all the observers who have made tests (see footnote, p. 433). During the absence of Dr Macrae on active service, Dr B. S. Platt acted as Secretary.

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Appendix A: Thiochrome Method for Estimating Vitamin B_1 in National Flour and Bread

The thiochrome test is based upon the work of Jansen [1936], who first adapted the oxidative formation of thiochrome to the determination of vitamin B_1. Two alternative techniques are here described, which have involved in their gradual evolution much labour and reduction of experimental difficulties; the one involves a visual and the other a photoelectric comparison of the formed thiochrome. That employing the visual comparison essentially follows the procedure of Harris & Wang [1941; see also Wang & Harris, 1942]; the photoelectric procedure is that of Booth [1940; 1942; see also Nicholls, Booth, Kent Jones & Ward, 1942]. Based upon the latter, there have been published already two reports on the vitamin B_1 control of national flour and bread by the Research Association of British Flour-Millers [1942] and the Ministry of Food [1942].

Jansen's work depended upon the previous findings: (*a*) by Peters that oxidation caused the production of blue fluorescent substances from vitamin B_1 [Peters, 1935]; (*b*) by Kuhn, Wagner-Jauregg, Klaverin & Vetter [1935] that a blue fluorescent sulphur-containing compound similar to dehydroaneurin could be isolated from yeast; this they called thiochrome; and (*c*) by Barger, Bergel & Todd [1935] that the latter (which they isolated and analysed) could be produced by the action of alkaline ferricyanide on aneurin. Its constitution was confirmed by synthesis by Todd, Bergel, Fraenkel-Conrat & Jacob [1936].

Jansen and his colleagues have used photoelectric methods for comparison of fluorescence; others use visual and still others such as Mukherji [1939] the Pulfrich photometer. Important steps have been the freeing of aneurin from biological combination by pepsin [Westenbrink & Goudsmit, 1938] and by takaphosphatase [Kinnersley & Peters, 1938] upon advice from Dr H. D. Kay. Valuable work upon the estimation of aneurin in foodstuffs was published by Pyke [1939] and by Houston, Kon & Thompson [1940]; see also Ritsert [1938]. An absorption step was used by Westenbrink & Jansen [1938] and by Hennessy & Cerecedo [1939]; for a further review see Hennessy [1941] and for a critique for fluorophotometers Loofbourow & Harris [1942].

Principle of method

The vitamin, after extraction and purification* by suitable methods, is oxidized to thiochrome, a blue fluorescent substance, which is estimated visually or by a fluorimeter. The methods for flour and bread described herein, which are based on practical experience and designed for speed and convenience of working, are not necessarily applicable to the estimation of vitamin B_1 in materials other than flour and bread.

Reagents. (Those marked [v] are required only for method employing visual comparison and those marked [p] only for method employing photoelectric comparison.)

Hydrochloric acid (A.R.): 0·02 *N* [v].
Hydrochloric acid (A.R.): 2% (w/v) [p].
Hydrochloric acid (A.R.): 10% (w/v) [v].
Sodium hydroxide (A.R.): 10% (w/v) [v].
Sodium hydroxide (A.R.): 20% (w/v) [v].
Sodium hydroxide (A.R.): 30% (w/v) [p].
Potassium ferricyanide (A.R.): 5% $K_3Fe(CN)_6$ (w/v).
Hydrogen peroxide solution: 20 vol. [v].
Sodium acetate-acetic acid buffer solution (*p*H 4·0): 36 parts of *M*/10 Na acetate (A.R.) and 164 parts of *M*/10 acetic acid (A.R.).
Bromocresol green solution: 0·04% [v].
Quinine sulphate (stock solution): 0·01 g. quinine sulphate (B.P.)/100 ml. 0·1 *N* H_2SO_4 [p].
Ethanol: 96% (redistilled till free from fluorescence).
Methanol: (redistilled till free from fluorescence) [v].
Isobutanol: (redistilled till free from fluorescence and shaken with water until saturated).
Toluene A.R.
Papain dry powder.
Takadiastase dry powder.

* A method of purification which completely obviates a 'blank' is one in which adsorbents are used; this method is recommended in U.S.A. and has received support in one laboratory in England.

Vitamin B_1 stock solution: 20 μg. vitamin B_1 hydrochloride (British National Standard for Vitamin B_1*) per ml. of 0·01 *N* HCl containing 25% (v/v) ethanol. For standardization, dilutions of this stock solution should be made with 0·01 *N* HCl on day of test.

General instructions

Identical laboratory conditions (especially in regard to temperature) should be secured for conversion of vitamin B_1 into thiochrome in both standard and unknown solutions. Test-tubes used in comparing the fluorescence of solutions by the visual method should be of non-fluorescent glass; the closed ends of the tubes should all be of the same shape; the tubes should be uniform in diameter, which should be such that 10 ml. occupy a column of 6–7 cm. For any one series of comparisons the diameters of the tubes should be identical.

Corks should be 'extracted' by refluxing for 1 hr. with ethanol, followed by 1 hr. with ether and finally by washing with isobutanol until the washings are non-fluorescent.

In order to avoid the introduction of fluorescent impurities, solutions should not come into contact with 'unextracted' corks, rubber (e.g. bungs or teats), or with tap greases containing blue fluorescent substances.

Only distilled water of high quality (preferably glass distilled) should be employed.

Solutions containing thiochrome should be kept away from bright light and comparisons of fluorescence made in a darkened room.

Method employing visual comparison of fluorescence

(1) *Extraction of the vitamin.* (*a*) Heat 2 g. of flour (or bread) with 15 ml. of 0·02 *N* HCl for 10 min. in a conical flask (50 ml.) on a boiling water bath. Stir the flask at intervals. Then add 5 ml. of Na acetate-acetic acid buffer (*p*H 4·0), mix and cool to approximately 40°. Using bromocresol green as external indicator check the *p*H; if necessary, adjust to *p*H 4·0 by the addition of either 10% HCl or 10% NaOH. To the pasty suspension add about 0·1 g. takadiastase and about 0·1 g. papain. Add a few drops of toluene as preservative and maintain at 40–45° for 16–18 hr. Cool to room temperature and add distilled water to make up to 25 ml. Centrifuge and retain the supernatant fluid. (The volume occupied by the undigested residue is small and is to be neglected.)

(*b*) For flour only, the following alternative procedure may be adopted. Make 2 g. flour into a cream with 25 ml. 2% HCl, allow to stand 16–18 hr. at room temperature, agitate and then centrifuge until the supernatant fluid obtained is only slightly cloudy.

(2) *Removal of fluorescent impurities by washing with isobutanol.* To 5 ml. of the supernatant fluid contained in a centrifuge tube (15 ml.) add a drop of 10% HCl, to ensure that the solution is more acid than *p*H 4·0, followed by 5 ml. of isobutanol. Stopper the tube with a cork, shake for not less than 1 min., centrifuge, remove the upper isobutanol layer, retaining the aqueous layer, the volume of which should be recorded.

(3) *Development of thiochrome from vitamin B_1 in the purified extract.* (*a*) Take three stoppered glass cylinders* (25 ml.) labelled A, B and C. Into A (the unknown) introduce 1·5 ml. of aqueous layer, add 2 ml. methanol, then 1 ml. 20% NaOH followed within 30 sec. by 0·15 ml. $K_3Fe(CN)_6$ solution, mixing thoroughly after each addition. Into B (the blank) measure 1·5 ml. aqueous layer, 2 ml. methanol and 1 ml. 20% NaOH. Into C (the control) put 1·5 ml. diluted standard vitamin B_1 solution containing 4 μg. of the vitamin followed by 2 ml. methanol, 1 ml. 20% NaOH and 0·15 ml. $K_3Fe(CN)_6$ solution, observing exactly the same procedure as for A.

To the mixture in cylinders A, B and C add 0·25 ml. H_2O_2, mix and allow to stand ½–1 min. Add 10 ml. isobutanol to each of the three cylinders, shake vigorously for 2 min. and allow the layers to separate. The upper isobutanol layer contains the thiochrome.

(*b*) In each case, draw off and discard the bottom aqueous layer; wash the isobutanol layer by shaking with 3 ml. distilled water for 1 min. Allow the layers to separate and transfer the upper layer to a dry conical flask. Then clarify by adding 2 ml. of ethanol.

(4) *Comparison of the fluorescence.* Measure 10 ml. of the clear isobutanol extract from the 'unknown' (A) and the 'blank' (B) into separate test-tubes. Compare in a dark room in front of the Wood's glass window of an ultra-violet lamp. Add the standard 'control' isobutanol extract (C) in small portions from a graduated pipette or burette to the 'blank' extract (B) until the fluorescence observed on looking down the tube matches exactly that of the 'unknown' (A). After each addition mix by stoppering with a cork and inverting. The tubes should be reversed frequently during the matching to avoid errors due to uneven illumination. Prior to the final matching an amount of isobutanol equal in volume to that of the 'control' isobutanol extract (C) added to the 'blank' (B) should be added to the unknown (A). It is advisable to use a Wratten 18A or similar filter in conjunction with Wood's glass to reduce errors due to substances with fluorescences of tints other than the purplish blue tint of thiochrome. (The H_2O_2 solution should have greatly reduced any non-specific yellowish fluorescence.) Matching must be related to the purplish blue tinted fluorescence of thiochrome, any other tint being ignored. Matching must be carried out rapidly since prolonged illumination increases eye fatigue, and the risk of photochemical destruction of thiochrome. Best comparisons are obtained by exposure and observation of 2–3 sec. periods, followed by periods of rest.

(5) *Calculation of results.* If x is volume in ml. of 'control' isobutanol extract (C) required and r_1 and r_2 the volumes in ml. of the extracts respectively before and after preliminary washing with isobutanol (para. 2), then the aneurin content of sample is

$$\frac{4x}{10} \times \frac{25}{1{\cdot}5 \times 2} \times \frac{r_2}{r_1} = \frac{10xr_2}{3r_1} \ \mu\text{g./g.}$$

Method employing photoelectric comparison of fluorescence

For use in the Spekker or other suitable fluorimeters, higher initial concentrations of flour or bread extracts may

* Obtainable by application to the Director of Biological Standards, National Institute for Medical Research, N.W. 3.

* Other types of reaction vessels may be utilized, e.g. glass-stoppered 1 oz. bottles or small separating funnels if the reagents are mixed by shaking, and boiling tubes if mixing is achieved by bubbling compressed air or nitrogen from a cylinder.

be required. In such cases, owing to the large bulk of solid matter (extracted flour or bread) present, it is necessary to carry out comparisons of standard against both the plain flour or bread, and flour or bread with a suitable aneurin addition. This dual comparison serves two purposes: (*a*) it makes allowance for substances which may be present and which interfere with the amount of fluorescent light which falls on the photocell by absorbing either the incident activating light or the emitted fluorescent light—in other words it serves as a recovery test—and (*b*) it enables the aneurin content of the extract to be related quantitatively to that of the flour or bread.

(6) *Extraction of the vitamin.* (*a*) The following procedure applies in particular to the Spekker fluorimeter but may readily be adapted to other fluorimeters. Take two 10 g. lots of flour or bread (A and A_1) in 100 ml. flasks; to A add 50 ml. acetate buffer from a graduated cylinder or burette and to A_1 a known amount of aneurin contained in a small volume of liquid, followed by a sufficient amount of acetate buffer to make the total volume of the additions 50 ml. A convenient amount of aneurin to add is 40μg. (2 ml. stock solution of standard), representing the addition of 4μg. aneurin to each gram of flour or bread being extracted. The suspensions of flour or bread should be free from lumps. To each flask add about 0·1 g. takadiastase and about 0·1 g. papain and a few drops of toluene as preservative and maintain at 40° overnight. Agitate and centrifuge and retain the supernatant fluids.

(*b*) For flour only, the following alternative is satisfactory. Take the two 10 g. lots of flour (as in 6 (*a*)); to one add 50 ml. 2% HCl and to the other the aneurin addition (as in 6 (*a*)) and sufficient 2% HCl to bring the total volume of the additions up to 50 ml. Allow to stand overnight at room temperature, agitate, centrifuge, and retain the supernatant fluids.

(7) *Removal of fluorescent impurities by washing with isobutanol.* This step is only infrequently necessary when using the photoelectric method. When, by visual inspection in ultra-violet light, the final isobutanol solution of thiochrome is seen to have strong non-characteristic fluorescence (usually white) superimposed on the characteristic purplish blue of the thiochrome, this step becomes necessary. It may be carried out exactly as in para. 2. Owing to dilution of the aqueous extract during the process, a factor r_1/r_2 (generally 11/10, see para. 5) must be applied in the final calculation of results.

(8) *Development of thiochrome from vitamin B_1 in the extracts.* Take three 6 × 1 in. boiling tubes, or alternatively stoppered vessels, labelled A, B and C. Into A and B, pipette 2 ml. of the plain flour or bread extract and into C, 2 ml. of the flour or bread extract containing added aneurin. To each tube add 2 ml. of methanol.

Then to tube A (the blank) add 1 ml. 30% NaOH solution and to tubes B and C, 1 ml. 30% NaOH followed within 30 sec. by 0·25 ml. $K_3Fe(CN)_6$ solution. The contents of the tubes are either kept constantly agitated by bubbling a stream of air or N_2 through them during the course of all additions or are mixed by shaking after each addition, according to whether boiling tubes or stoppered vessels are employed. Oxidation is allowed to proceed for a minimum of 1 min., after which 25 ml. isobutanol are added and the bubbling is continued for 2 or 3 min., or the stoppered vessels are shaken vigorously for about 1 min.

The aqueous and isobutanol layers are allowed to separate for about 15 min. to reduce turbidity, 1 ml. ethanol is added to each tube and is mixed carefully into the upper (isobutanol) layer (by means of the stream of air or a glass rod). The isobutanol layer is then decanted into the fluorimeter cell. Slight turbidity of this solution has not been found to influence the fluorimeter results.

(9) *Comparison of the fluorescence.* When the Spekker fluorimeter is used the fluorescence of the unknowns should be measured in terms of a standard which is stronger than the unknowns. As thiochrome is rather unstable in ultra-violet light, it is unsuitable as a standard, and quinine sulphate has therefore been adopted for the purpose. A suitable strength of solution is 1μg. quinine sulphate/ml. 0·1 *N* H_2SO_4, and this can be made up monthly from the stock solution (kept in the dark) of 100 times this strength. The 'standard' cell is, however, freshly filled with the quinine solution daily. The instrument is calibrated against the quinine standard by means of a series of aneurin standards (e.g. 0·1–3·0μg. for the Spekker), which have been prepared by oxidizing standard solutions of aneurin by precisely the same technique as is employed for flour or bread extracts. A standard curve is plotted relating E to μg. of aneurin, and readings are taken with the three extracts prepared above. If it happens that the concentrations suggested are outside the limits of the instrument, the estimation should be repeated, using a more suitable amount of extract, since dilution of the thiochrome layer is not permissible.

(10) *Interpretation of results.* The indicated vitamin B_1 content of the flour or bread extract minus the 'blank' is not precisely the vitamin B_1 content of the aliquot of extract, owing to the possibility that interference may lower the fluorescence. This is allowed for (at the same time relating the flour or bread extract to the flour or bread itself) by the use of the flour (or bread) plus aneurin values. It is known that the difference between the two flours or breads is precisely 4μg. aneurin/g. Then, to take a concrete example, if an aliquot of the plain flour minus blank showed 1·5μg. and the flour plus aneurin minus blank showed 3·5μg. per aliquot, it follows that the aneurin content of the flour is

$$\frac{4}{3{\cdot}5 - 1{\cdot}5} \times 1{\cdot}5 \text{ or } 3\mu\text{g./g.}$$

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An Azo Colour Reaction for the Determination of Vitamin B_1 in National Flour

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Jansen & Donath [1926 *a*, *b*; 1927] reported that vitamin B_1 concentrates gave a Pauly colour reaction with diazotized sulphanilic acid. Kinnersley & Peters [1927; 1928], Windaus, Tschesche, Ruhkopf, Laquer & Schultz [1931] and Van Veen [1932], however, considered that vitamin B_1 did not give a Pauly reaction as ordinarily carried out or as modified by Koessler & Hanke [1919]. By increasing the alkalinity of the reaction as carried out by Koessler & Hanke, Kinnersley & Peters [1927] found that vitamin B_1 gave a pink colour instead of a yellow one, but this was tinged with yellow and faded gradually. Later, Kinnersley & Peters [1934] found that the pink colour could be intensified and stabilized by the addition of formaldehyde but that the yellow colour still persisted. Barger, Bergel & Todd [1935] suggested that it is the free amino group which reacts with diazonium salts. Todd & Bergel [1936], however, consider the formaldehyde-azo colour reaction of Kinnersley & Peters depends on the presence of a β-hydroxy-ethyl group in position 5 and a hydrogen atom in position 2 of the thiazole nucleus.

Diazotized *p*-aminoacetophenone was used by Prebluda & McCollum [1936; 1937; 1939] as a qualitative test for vitamin B_1 and by Platt & Yang [1937] and Yang & Platt [1939] for the quantitative estimation of vitamin B_1 in biological material, the reaction being carried out in the presence of the clay on which they adsorbed the vitamin B_1. Willstaedt & Bárány [1938] and v. Euler [1940–1] employed diazotized 2:4:dichloroaniline.

Melnick & Field [1939] also used Prebluda and McCollum's reaction in the quantitative estimation of vitamin B_1. They carried out a preliminary adsorption of the vitamin on 'Decalso', eluted it with hot acid KCl solution and developed the colour in the presence of phenol, which, they state, makes the reaction more sensitive, has a protective action on the oxidation of the vitamin B_1 in alkaline solution and prevents the inhibition of colour reaction caused by various substances. Emmett, Peacock & Brown [1940] adopted Melnick & Field's procedure except that they adsorbed the vitamin B_1 on 'Superfiltrol' instead of on 'Decalso', and carried out the colour reaction in the presence of the adsorbate, on which the dye was quantitatively adsorbed and subsequently eluted with ethanol. Auerbach [1940] has simplified Prebluda & McCollum's reagents and applied the reaction to the quantitative estimation of vitamin B_1 in certain pharmaceutical preparations, omitting the use of any adsorbent.

Prebluda & McCollum [1939] tested the specificity of the *p*-aminoacetophenone reaction, using several pyrimidine and thiazole derivatives. Some of the thiazole derivatives gave purple-red compounds with the alkaline diazo reagent. The free thiazole portion of the molecule reacted much more slowly and less completely than vitamin B_1 itself. They concluded that the presence of the methylene bridge between the thiazole and pyrimidine portions of the molecule was necessary for complete reaction. Melnick & Field found that degradation products of vitamin B_1 did not react. This was true after sulphite cleavage, which yields intact pyrimidine and thiazole derivatives; after the action of alkali and heat, which results in the opening of the thiazole ring; after simple deamination, and after oxidation to the thiochrome stage.

Kirch & Bergeim [1942] have recently described a colour reaction for vitamin B_1 with diazotized ethyl *p*-aminobenzoate, the dye being subsequently dissolved in *iso*-amyl alcohol. They claim that preliminary adsorption is unnecessary under their con-

ditions for the determination of vitamin B_1 in urine, but they have not applied the reaction to any other biological material. Using pure vitamin B_1 solutions, we have found this colour reaction to be much less sensitive than with diazotized *p*-aminoacetophenone using the procedure described below. We have attempted to apply their reaction to flour extracts and to 'Superfiltrol' adsorbates of flour extracts. In both cases, the colour reaction was completely inhibited, a bright yellow colour being extracted by the solvent.

We have recently examined the procedure of Melnick & Field and find that although their method of adsorption and elution is fairly satisfactory, it is a tedious and complicated procedure. A more simple method has been devised which was finally adopted for the estimation of vitamin B_1 in national flour.

EXPERIMENTAL

Extraction of vitamin B_1

The vitamin has been extracted from flour with 0·5 *N* HCl and, in order to be sure of complete extraction, it has been found advisable to stir up the flour suspension at intervals over a period of several hours and finally to leave the suspension to settle overnight. Since flour contains a negligible amount of phosphorylated vitamin B_1, preliminary enzymic digestion has been omitted.

It has been found that during extraction the whole of the vitamin B_1 passes into the liquid phase. Thus, in calculating the vitamin B_1 content of flour, it is permissible to take the volume of the extracting fluid as the volume of the flour extract, so that if, as in the following procedure, 25 g. flour are extracted with 100 ml. 0·5 *N* HCl, the vitamin B_1 contained in 40 ml. of the filtered extract is equivalent to 10 g. original flour.

Adsorption of vitamin B_1 from the flour extract

Quantity of adsorbent. This has been kept as small as possible so as to minimize the amount of dye precipitated on it at a later stage in the process. One adsorption of flour extract (=10 g. flour) on 50 mg. adsorbent removed approximately 90% of the vitamin B_1, a second adsorption on a further 50 mg. removing all the remaining vitamin B_1 from solution. Two successive adsorptions on 50 mg. 'Superfiltrol' have consequently been employed.

pH for adsorption. Quantitative adsorption of the vitamin B_1 from flour extracts was obtained without any adjustment of *p*H.

Time and conditions for adsorption. Shaking for a total period of 1 hr. on a tumbler rotating at approximately 60 r.p.m. has been found adequate for complete adsorption of the vitamin B_1 from flour extracts.

Adjustment of the adsorbate prior to the colour development

pH adjustment. Prebluda & McCollum [1939] advocated adjusting the *p*H to 5·0–6·0 immediately before carrying out the reaction. Melnick & Field [1939] give experimental evidence for adjusting the *p*H of vitamin B_1 in alcoholic solution to 7·0, but in practice add alkali until their solution is blue to thymol blue. Emmett *et al.* [1940] adjusted the *p*H in the presence of alcohol to 7–8.

We found that solutions at *p*H from 2–9, adjusted in the presence of alcohol, gave erratic results. Consistent and reproducible results were, however, obtained from *p*H 4–7, if the *p*H adjustment was made immediately before the addition of the ethanol.

Concentration of ethanol. The presence of a high concentration of ethanol in the final stages of the reaction is advantageous as it helps to elute vitamin B_1 from the adsorbent, stabilizes it, intensifies the colour produced [Melnick & Field, 1939] and also keeps the dye in solution. The concentration of ethanol (40%) cannot be exceeded, otherwise salts from the alkaline diazo reagent are precipitated.

Factors affecting the colour reaction

Preparation of alkaline diazo reagent. An alkaline reagent similar to that used by Kinnersley & Peters [1927] was incorporated in the reagents of Prebluda & McCollum [1939] and was used unmodified by Melnick & Field [1939], except that the latter workers carried out the colour reaction in the presence of phenol and ethanol. Prebluda & McCollum allowed the alkali and diazo reagent to react for at least 15 min. before carrying out the colour reaction. This procedure, we have found, diminishes considerably the colour produced.

Like Koessler & Hanke, we have found it necessary to add the alkaline diazo reagent to the alcoholic suspension of vitamin B_1 exactly 1 min. after mixing the diazo reagent and alkali.

The relative concentrations of alkali and diazo reagent have been found to be very critical. Employing the same reagents as Prebluda & McCollum, we have ascertained the effect on the colour development of varying the relative concentrations of diazo reagent and alkaline reagent. The two curves in Fig. 1 show the effects of (*a*) varying the amount of alkaline reagent from 1·0 to 5·0 ml., keeping the volume of diazo reagent constant at 2·5 ml., and (*b*) varying the amount of diazo reagent from 1·0 to 5·0 ml., keeping the volume of alkaline reagent constant at 2·5 ml. In both cases the reaction was carried out in the presence of a flour adsorbate, treated as described below, the colorimeter readings/100 mg. wet adsorbent being plotted against the volume of reagent (Fig. 1).

From these results it was concluded that for estimating the vitamin B_1 contained in 10 g. national flour, 2·5 ml. diazo reagent to 2·25 ml. alkaline reagent are required.

In the following method, the concentrations of acid *p*-aminoacetophenone solution and the alkaline reagent have for convenience been adjusted so that 2 ml. of both diazo reagent and alkali contain the optimal amounts of the reactants. The final diazo mixture is considerably less alkaline than that used by Prebluda & McCollum.

Presence of phenol. According to Melnick & Field, phenol intensifies the colour produced. Using our reagents we have, however, obtained much better colour development than did Melnick & Field employing phenol in addition. We consider the presence of phenol unnecessary.

Reaction time. Melnick & Field state that it is necessary to leave the reaction mixture overnight for complete colour development. Using the procedure described below, the maximum is reached at the end of 1 hr.

Extraction of the dye. Toluene is a more efficient solvent for the dye than xylene (used by Melnick & Field [1939]) in the presence of the adsorbent. The colour in toluene is, moreover, much more stable in bright daylight. Toluene, however, volatilizes more rapidly than xylene and prefrom the flour adsorbate reaction mixture alone with that from a flour extract to which vitamin B_1 had been added immediately before the colour reaction was carried out. The tints of the standard and unknown were identical by this procedure.

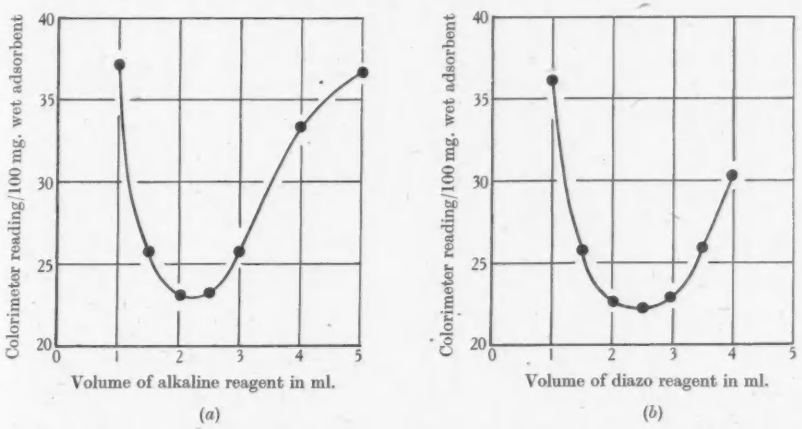

Fig. 1. The colour developed when flour adsorbate reacts with the diazo reagent in alkaline solution under different conditions: (*a*) with constant vol. (2·5 ml.) of diazo reagent, (*b*) with constant vol. (2·5 ml.) of alkaline reagent.

cautions have to be taken to prevent evaporation at any stage.

Emmett *et al.* [1940] adsorbed the dye quantitatively on 'Superfiltrol' and subsequently eluted it with ethanol. In order to simplify and shorten the procedure, we have attempted to keep most of the dye in solution by using a high concentration of ethanol, and have extracted the dye directly from solution by toluene. Extraction of the dye from the reaction mixture was found to be much more difficult if delayed until maximum colour development is reached, since, by that time, most of the dye is carried down on the adsorbent. Toluene was therefore added 20 min. after the beginning of the reaction. This procedure also accelerated the attainment of full colour development, which is never reached if extraction of the dye is begun during the initial 10 min.

Choice of standard

In early tests, the dye derived from flour extracts was compared with that from a standard vitamin B_1 solution, the colour reaction being carried out in the presence of 'Superfiltrol'. In the absence of 'Superfiltrol', the colour extracted from the vitamin B_1 reaction mixture was more purple than that extracted from a flour reaction mixture.

Values of more than 100% for the recovery of vitamin B_1 were obtained when the vitamin was added to flour extracts before adsorption. Similar values were obtained if vitamin B_1 in solution was added after adsorption and immediately before the colour reaction was carried out. In both cases, i.e. with 20μg. vitamin B_1 added before and after adsorption, a value of 22·6μg. vitamin B_1 was obtained. It thus appears that the presence of the flour adsorbate intensifies the colour produced, and it was therefore decided to compare the colour of the dye extracted

Method adopted

Reagents

(1) 0·5N HCl (approx.).

(2) 2% NaOH (w/v).

(3) Alkaline reagent: 3% $NaHCO_3$ (w/v) in 0·6N NaOH.

(4) *p*-Aminoacetophenone solution: 0·8% *p*-aminoacetophenone in 3·5 HCl (w/w). Keep in a dark bottle in the cold.

(5) 4·4% $NaNO_2$ (w/v) (Analar). Keep in the cold, for not longer than 1 month.

(6) Diazo reagent: Measure 1·5 ml. *p*-aminoacetophenone solution into a 50 ml. volumetric flask and cool to 0–5°. Add 1·5 ml. $NaNO_2$ solution (previously cooled to 0–5°), mix and leave in the cold for 10 min. Add a further 6 ml. $NaNO_2$ solution, mix and leave for 10 min. Make up to 50 ml. with ice-cold distilled water and allow to stand at least 15 min. before use. (This reagent should be prepared on the day of the test and must be kept at 0–5°.)

(7) 'Superfiltrol' (activated clay).

(8) Toluene (rectified).

(9) 60% C_2H_5OH (v/v) in distilled water.

(10) Standard vitamin B_1 solution: (*a*) Stock solution containing 150μg. vitamin B_1 (hydrated—4·65% moisture)/ml. in 0·01N HCl containing 25% ethanol. (*b*) Solution for use: Dilute the stock solution with distilled water on the day of the test so that it contains 30μg. vitamin B_1/ml.

Extraction of vitamin B_1 from flour and treatment of the adsorbate. (i) Stir 25 g. national flour into 100 ml. 0·5*N* HCl in a beaker, and leave at room temperature throughout the day preceding the test. Stir thoroughly at intervals and leave to stand overnight. Pour off the supernatant fluid and filter through a No. 1 Whatman filter paper (a satisfactory extract cannot be obtained by centrifuging).

(ii) Pipette 40 ml. of the filtrate into a bottle of 100–150 ml. capacity, provided with a glass stopper. Add 50 mg. activated clay, stopper and shake for 30 min. (a tumbler rotating at 60 r.p.m. is satisfactory). Transfer the contents of the bottle to a tapering 50 ml. centrifuge tube, wash out of the bottle as much of the adsorbate as possible with 5 ml. 0·5*N* HCl and add the washings to the contents of the centrifuge tube. Centrifuge until the adsorbate is well packed. Pour the supernatant fluid back into the bottle and shake for a further 30 min. with another 50 mg. activated clay. Transfer the contents to the centrifuge tube containing the first adsorbate and centrifuge. Discard the supernatant fluid. Wash out the bottle with five successive portions of 5 ml. 0·5*N* HCl, transfer the washings to the centrifuge tube containing the adsorbate and resuspend the adsorbate in the washings. Centrifuge again and discard the supernatant fluid.

(iii) Add to the washed adsorbate 1 ml. distilled water, 2 drops bromo-cresol-green and adjust to *p*H 5 (approx.) by the dropwise addition of 2% NaOH, keeping the adsorbate in suspension by stirring with a glass rod. Pour the suspension through a small glass funnel into a 25 ml. glass-stoppered measuring cylinder. Wash out the centrifuge tube with five successive portions of 2 ml. 60% ethanol, adding the washings to the suspension in the cylinder. Proceed immediately with the following step.

Development of colour. To 2 ml. NaOH-$NaHCO_3$ solution contained in a dry test-tube add 2 ml. diazo reagent. Invert to mix and, exactly 1 min. after mixing, add to the suspension of adsorbate in the measuring cylinder. Stopper and mix by inversion at intervals of a few minutes. After 20 min. introduce 5 ml. toluene into the cylinder. Restopper and continue to mix by inversion (done gently so as to avoid emulsion formation) at frequent intervals for a total period of 1 hr. or longer. Withdraw the toluene layer into another stoppered cylinder containing 10 ml. 0·5*N* HCl and wash by inversion. Transfer the washed toluene layer to a 5 ml. tapering centrifuge tube, shake in a small amount of anhydrous Na_2SO_4, mix and close the tube with a well-fitting cork. Centrifuge for a very short time, avoiding any risk of loss of solvent by evaporation.

Preparation of standard solution. To a second washed adsorbate, prepared as described in (i) and (ii), add 1 ml. of diluted standard vitamin B_1 solution and 2 drops of indicator and continue from this stage exactly as before with one difference, i.e. extract the dye with 10 ml. toluene instead of 5 ml.

Comparison of solutions. The two solutions are compared in a Duboscq type of colorimeter fitted with cups having a stem capacity of 5 ml.

Accuracy of method

(*a*) *Solutions of pure vitamin B_1.* The accuracy was determined on amounts of 20, 30 and 40 μg. pure vitamin B_1 in solution. The procedure adopted was the same as that described above for flour extracts except that preliminary adsorption on 'Superfiltrol' was omitted and it was found necessary to extract the dye with toluene after 60 min. instead of after 20 min., since the colour production is retarded in the absence of 'Superfiltrol'. The accuracy of the estimations (in triplicate) carried out by three different observers is set out in Table 1.

Table 1. *Statistical analysis of the results of vitamin B_1 determinations*

Amount of pure vitamin B_1 in test sample (μg.)	20		30		40	
Average of 9 observations (μg.)	20·11		30·12		39·98	
Standard deviation ...	0·4106		0·6099		1·0208	
Value of *P*	0·95	0·99	0·95	0·99	0·95	0·99
Limits of error:						
For determinations in duplicate	2·8	3·7	2·8	3·7	3·5	4·7
For determinations in triplicate	2·3	3·0	2·3	3·0	2·9	3·8
For determinations in quadruplicate	2·0	2·6	2·0	2·6	2·5	3·3

(*b*) *Extracts of national flour.* A number of results for the vitamin B_1 contents of national flours, using our azo method as described in this paper, obtained by different observers, have been published in a Medical Research Council report [1943]. The accuracy of the results obtained compares favourably with that of the thiochrome method.

SUMMARY

A simple colorimetric method is described for the routine estimation of vitamin B_1 in national flour using diazotized *p*-amino-acetophenone. The method, with minor modifications, can be used for the estimation of pure vitamin B_1.

We are indebted to Dr Katharine Coward for her statistical analysis of the results set out in Table 1.

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A Quantitative Method for the Determination of Uroporphyrin in Pathological Urines

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The chief difficulty attending the isolation of porphyrins from urine and their separation from one another is the relatively sparing solubility of these pigments in organic solvents. Coproporphyrins I and III are extractable by the acetic acid-ether technique discovered by Saillet [1896] and adopted by Fischer [1926] and may thus be quantitatively determined. For preparative purposes, although large volumes of solvent are required, a high degree of purification may be achieved by repeated transferences between ether and dilute HCl. Uroporphyrin III is similarly extractable by ethyl acetate from solutions adjusted to a *p*H of 3·0–3·2 [Waldenström, Fink & Hoerburger, 1935], but again large volumes of solvent are required for this laborious process, which is not suitable for quantitative purposes. Uroporphyrin I is not extracted by any known solvent immiscible with water.

Recourse must, therefore, be had to adsorptive procedures for the concentration and purification of the uroporphyrins prior to their spectrophotometric or fluorimetric determination. Many different materials have been proposed as suitable adsorbents for porphyrins. They include Ca and Pb phosphates, alumina, talc, $BaSO_4$, etc., and Waldenström [1935] has elaborated a chromatographic procedure for the adsorption of porphyrins from urine upon alumina. In this, as in all other procedures, adsorption takes place at or near the isoelectric point of the porphyrin. At this reaction the greater part of the dark brown or red water-soluble pigment of unknown constitution, always present in porphyrinuric urines, is also adsorbed, so that from a quantitative point of view the operation is of little value, and further purification of the uroporphyrin fraction is difficult. Attempted purification of the porphyrins from urine by acidifying with acetic acid [Nebelthau, 1899] is not always successful, and in any precipitate which forms coproporphyrin is mixed with uroporphyrin.

While searching for a new adsorptive technique, it was discovered that kieselguhr has the very desirable and remarkable property of adsorbing both coproporphyrins and uroporphyrins from solution *in dilute HCl* whilst rejecting almost entirely the dark, brown-red, non-porphyrin pigment. Elution by suitable buffers brings about a fairly sharp separation of the coproporphyrins from the uroporphyrins, but it has not been found possible to separate by this means the I and III isomers of either pigment, no doubt owing to the fact that their isoelectric points are so similar. Based upon the kieselguhr technique, a good quantitative method has been worked out for the determination of total porphyrin, and hence of uroporphyrin, in porphyrinuric urines, thus for the first time enabling

conditions such as acute idiopathic porphyrinuria to be subjected to accurate quantitative examination.

METHODS

Separation of pure coproporphyrin from uroporphyrin

The pigments used had been prepared from natural sources and purified by recrystallization of their methyl esters. These were saponified and the free porphyrins brought into solution in 5% HCl (w/w). Tswett columns were prepared from acid-washed kieselguhr (B.D.H.) suspended in 5% HCl (w/w) and the behaviour of the pigments studied individually on filtering their solutions through the columns, and on developing with buffer solutions. All porphyrins tested were strongly adsorbed from the acid solutions. Table 1 summarizes the results obtained with

Table 1. *Behaviour of coproporphyrins and uroporphyrins adsorbed on kieselguhr*

Developer	Effect on porphyrin: Uroporphyrins I or III	Effect on porphyrin: Coproporphyrins I or III
20% HCl	Nil	Nil
50% acetic acid	Nil	Nil
Glacial acetic acid	Slow broadening of band	Slow broadening of band
Water	Nil	Nil
M Citrate buffer *p*H 3·2	Nil	Nil
M Acetate buffer *p*H 4·0	Nil	Nil
M Acetate buffer *p*H 4·5	Nil	Nil
M Acetate buffer *p*H 4·7	Broadening of band	Nil
M Acetate buffer *p*H 5·0	Broad ring passing slowly downwards	Nil
M Acetate buffer *p*H 5·25	All porphyrin removed	Very slow broadening of band
M Acetate buffer *p*H 5·5	—	Rapid broadening
M Phosphate buffer *p*H 6·5	—	Rapid broadening
M Phosphate buffer *p*H 7·15	—	All porphyrin removed

*p*H's measured by glass electrode.

the developers. A mixture of uroporphyrin III and coproporphyrin I was adsorbed on a column from acid solution as previously described and then washed thoroughly with water. Acetate buffer of *p*H 5·25 was then filtered through the column, when the uroporphyrin passed readily downwards, leaving a sharply defined zone containing the coproporphyrin at the top of the column. The eluate contained no ether-soluble pigment. The coproporphyrin was finally removed from the column by a phosphate buffer of *p*H 7·15.

Chromatographic separation of uroporphyrin from urine of acute porphyria

The specimen of urine, which was nearly black in colour, was passed during a period of severe attack by a man suffering from acute idiopathic porphyria. HCl was added to a concentration of 1% (w/w) and the mixture filtered through a column of kieselguhr. The deeply coloured filtrate was free from porphyrin. The column was washed well with 1% HCl (w/w) and then with glacial acetic acid, which removed the remaining dark brown pigment together with a little porphyrin detectable by fluorescence in ultra-violet light. After thorough washing with water, the uroporphyrin was then removed by acetate buffer of *p*H 5·25 and obtained as a deep red solution substantially free from other pigments. Upon adjustment of the *p*H to 3·0, the porphyrin precipitated readily, leaving a pale straw-coloured supernatant liquor. The precipitate was collected on the centrifuge, dried and esterified in the usual way. The ester crystallized from hot chloroform-methanol in fine microscopic needles, M.P. 258°.

The adsorption technique has also been used to purify the crude precipitate of mixed porphyrins obtained from a porphyrinuric urine by acidification with acetic acid. The mass was dissolved in dilute alkali and the *p*H adjusted to 5·25. Kieselguhr was then added and the mixture shaken vigorously and centrifuged. The adsorbent retained coproporphyrin, but little, if any, uroporphyrin. It is doubtful whether the method would be so effective were the proportion of coproporphyrin to uroporphyrin not so small (in the above case it was approximately 1:20), but by repeated shaking or filtration through a column it should be possible to remove substantially all coproporphyrin from uroporphyrin solutions by this simple expedient.

A quantitative method for the determination of uroporphyrin

The property possessed by kieselguhr of adsorbing uroporphyrin strongly from dilute acid solutions but of retaining very little of the accompanying urinary pigments provides the basis of a relatively simple method for the determination of uroporphyrin. The studies to be detailed below were carried out upon some specimens of urine from a case of acute idiopathic porphyria, and for his kindness in putting this material at my disposal I wish to thank Dr G. Discombe of St Bartholomew's Hospital.

Preliminary treatment of the urine. Waldenström & Vahlquist [1939] have shown that in acute porphyria a pigment precursor, porphobilinogen, is excreted, which is rapidly converted into porphyrin when the urine is acidified and warmed on the water bath [cf. Willenbücher, 1942]. During this heating, however, the urine becomes very dark owing to the formation of other, non-porphyrin, pigments and it has been found that the same full production of porphyrin is achieved without such undesirable accompaniment by rendering the specimen acid to a concentration of 1% HCl (w/w) and leaving it at room temperature overnight.

A known volume (usually 1–2 ml.) of urine containing approximately 5–50μg. of porphyrin is thus acidified, diluted with 1% HCl (w/w) to 5 ml. and left overnight. About 0·7 g. of acid-washed kieselguhr is then added, the tube stoppered, shaken vigorously for about 1 min., and then centrifuged for 5 min. The kieselguhr packs well, allowing the brown supernatant liquid to be decanted. Control examination of this liquid by ultra-violet light should show only a greenish-blue fluorescence unmixed with red, thus indicating that the quantity of adsorbent used has been sufficient.

Elution and quantitative determination of porphyrin

The kieselguhr is now washed by stirring up and centrifuging successively twice with 1% HCl (w/w) and once with H_2O.* The porphyrin is then eluted completely by three successive washings with 0·1 *N* NaOH of about 7 ml. each,

* From some urines, washing with 50% acetic acid removes a further small quantity of fluorescent material without loss of porphyrin.

and centrifuging for 15–20 min. The eluates may still contain fine particles of kieselguhr in suspension, but these are disregarded since, when all the eluates are combined, made up to a volume of 25 ml. and left overnight, the turbidity subsides completely, leaving a clear solution which may be pipetted off. From this, a suitable dilution, 1/5 or 1/10, is prepared with 0·1 *N* NaOH, such that the final porphyrin concentration is near the optimal figure of 0·25 μg./ml., and the exact concentration is determined fluorimetrically in the fluorescence comparator [Rimington, 1943].

This determination gives the total porphyrin. Coproporphyrin is conveniently determined, by the acetic acid-ether extraction method and fluorimetric analysis, in a portion of the eluate, or in the urine itself. By subtraction the quantity of uroporphyrin is calculated. It has been found more convenient to elute the total porphyrin with dilute alkali rather than to attempt a two-stage elution and separation of coproporphyrin from uroporphyrin by means of buffers. Errors due to the effect of salts upon fluorescence intensity are thereby eliminated, and, furthermore, quantitative elution by buffers was found to be rather a tedious process, requiring relatively large volumes of fluid.

Recovery of added uroporphyrin was quantitative, and the error of the method, judged by results of repeated determinations, is estimated at less than 10%. The following example may be quoted in illustration:

Urine specimen 26. (Coproporphyrin by ether extraction 147 μg./100 ml.)

		Uroporphyrin mg./100 ml.
(1)	2 ml. urine + 2 ml. H_2O + 1 ml. 5% HCl (w/w), worked up immediately	0·96
(1*a*)	2 ml. urine + 2 ml. H_2O + 1 ml. 5% HCl (w/w), worked up immediately	1·08
(2)	2 ml. urine + 2 ml. H_2O + 1 ml. 5% HCl (w/w), heated on water bath for 1 hr.	1·72
(3)	2 ml. urine + 2 ml. H_2O + 1 ml. 5% HCl (w/w), left overnight at room temp.	1·67
(3*a*)	2 ml. urine + 2 ml. H_2O + 1 ml. 5% HCl (w/w), left overnight at room temp.	1·77
(4)	1 ml. urine + 2 ml. H_2O + 1 ml. (= 20 μg.) uroporphyrin III + 1 ml. 5% HCl (w/w), left overnight at room temp. Recovery of added uroporphyrin 109%	
(5)	100 ml. pooled eluates corresponding to 8 ml. urine (= 12 μg. coproporphyrin) extracted by ether, etc. Coproporphyrin in extract 12·4 μg. Recovery 103%	

RESULTS

Excretion of uroporphyrin and coproporphyrin in a case of acute idiopathic porphyria

The patient was a young man aged 19, under the care of Dr Geoffrey Evans of St Bartholomew's Hospital, for whose great kindness in allowing me to examine the urines and publish my biochemical findings, together with relevant data from the clinical record, I am very greatly indebted. He was admitted in April 1942, and presented the typical clinical picture of acute idiopathic porphyria. The prominent features of his condition were malaise and marked asthenia, complicated by peripheral neuritis and muscular wasting. Relapses were characterized by a return of abdominal pain, loss of appetite and nausea. He suffered an attack of pain on 25 May, but thereafter improved until 19 August which marked the onset of another sharp attack.

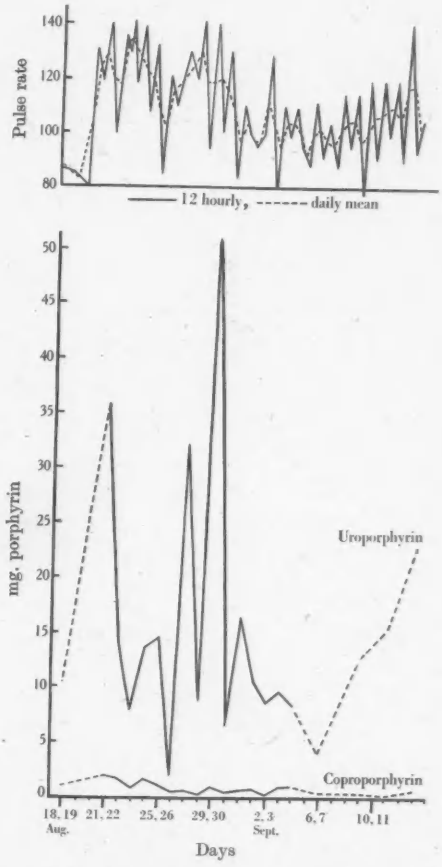

Fig. 1. Daily urinary porphyrin excretion and pulse rate in case of acute idiopathic porphyria. — Consecutive daily determinations. – – – Determinations at intervals of 48 hr. or more.

Urine specimens (24 hr. collections, for arranging which I am much indebted to Dr G. Discombe) were examined on the 12/13 August and 21/22 August, and thereafter daily until 5 September. This period embraced a typical attack. As the

patient's condition was improving by 5 September, analyses were performed only at 48 hr. or 72 hr. intervals until 14 September, when the investigation concluded.

Of most urine specimens, 1 or 2 ml. sufficed for the estimation of porphyrin, but of the weaker specimens 3 ml. were required, and in the case of those very rich in porphyrin 0·5 ml. was found to be ample. Table 2 presents the analytical results, together with notes upon the colour of the urines and the clinical condition of the patient. A comparison between the levels of uroporphyrin and coproporphyrin excretion from day to day and the 12-hourly records of the pulse rate (full line) and estimated 24-hourly fluctuations in pulse rate (dotted line) is set forth in Fig. 1.

of uroporphyrin follows the excursions of the pulse rate. There is no constancy in the ratio between coproporphyrin and uroporphyrin excreted or between total porphyrin and urine volume (see Table 2), although it would appear that the fluid output modifies slightly the output of pigment.

The urinary porphyrins excreted by this case were identified as uroporphyrin III, octamethyl ester M.P. 258°, and coproporphyrin III, tetramethyl ester M.P. 140°, remelt 163°, together with a small quantity of coproporphyrin I.

SUMMARY

1. Coproporphyrins and uroporphyrins of both series I and III are adsorbed by kieselguhr from

Table 2. *Case: 'Peter Beresford'. Analysis of urines for porphyrin*

Date	Colour of urine	Total volume ml.	Copro-porph. μg./ 100 ml.	Total copro-porph. mg.	Uro-porph. mg./ 100 ml.	Total uro-porph. mg.	Clinical condition
9/10 June	Nearly black	1420	30·6	0·435	1·9	27·0	Last attack of pain 25 May, condition improving
1/2 Aug.	Light red-brown	—	60·5	—	2·58	—	
12/13 Aug.	Dark	—	64·5	—	1·6	—	
18/19 Aug.	Light brown	1240	76·9	0·954	0·85	10·5	Pulse rate 80
21/22 Aug.	Dark	1915	108	2·068	1·87	35·7	Sudden rise of P.R. to 128 on 20th
22/23 Aug.	Deep brown	1860	100	1·860	0·76	14·14	P.R. 136
23/24 Aug.	Greenish brown	680	124	0·843	1·18	8·0	P.R. fell gradually to 85 on 23rd. Rose again to 110 on 25th; 120–130 on 26th; 140 on 27th a.m.; fell to 95 by p.m.
24/25 Aug.	Golden brown	1160	139	1·612	1·18	13·69	
25/26 Aug.	Red-brown	850	147	1·250	1·69	14·37	
26/27 Aug.	Dark purplish brown	930	68·4	0·657	0·293	2·81	
27/28 Aug.	Dark brown	900	74·4	0·728	3·264	31·93	P.R. 140 on 28th a.m., fluctuating, but fell to 85 by 30th. Patient lost 6¼ lb. since 9 Aug.
28/29 Aug.	Dark brown	182	132	0·251	4·718	8·96	
29/30 Aug.	Very dark	720	130	0·969	6·745	50·25	
30/31 Aug.	Dark brown	815	58	0·487	0·775	6·51	P.R. about 100
31 Aug./1 Sept.	Dark brown	1420	38·7	0·568	1·128	16·58	P.R. rose to 128 on 1st, falling to 80 on 2nd
1/2 Sept.	Dark brown	1100	71·3	0·806	0·954	10·78	
2/3 Sept.	Dark brown	1490	18	0·277	0·547	8·42	Attack subsided, according to P.R., on 16 Sept., when the rate returned to the normal range except for occasional rises to about 130. Steady slow improvement has continued since 18 Sept.
3/4 Sept.	Light brown	1810	49·5	0·923	0·501	9·44	
4/5 Sept.	Light brown	1920	49·5	0·980	0·416	8·24	
6/7 Sept.	Light brown	2290	18	0·425	0·165	3·89	
9/10 Sept.	Light brown	1780	22·7	0·415	0·705	12·90	
11/12 Sept.	Light brown	2120	11·6	0·254	0·716	15·54	
13/14 Sept.	Light brown	2040	35·2	0·739	1·096	23·02	

No interpretation of the clinical findings or discussion of the clinical aspects of the case is intended here; the record of the pulse rate is merely given as an accompaniment to the porphyrin determinations which illustrate the potential usefulness of the kieselguhr method. With such a relatively simple and rapid technique in one's hands, it is now possible to subject the attacks and remissions of acute porphyria to a quantitative examination, and seek to gain further insight into the pathogenesis of this disease. Whilst the correspondence is not exact, it may be said, from an inspection of Fig. 1, that, in general, the quantitative excretion

acid solution (1% HCl (w/w)), and a separation of the porphyrins, but not of individual isomers, may be effected by developing the chromatogram with acetate buffer of *p*H 5·25. Uroporphyrins I and III are readily eluted.

2. Improvements in the preparative method of obtaining uroporphyrin from pathological urine are based upon this observation.

3. A quantitative procedure for determining the total porphyrin in urine, and hence the uroporphyrin by subtraction of the independently determined coproporphyrin, is described and its application to the analysis of consecutive 24 hr. samples

from a case of acute idiopathic porphyria, during an attack, is recorded. The provision of a quantitative technique for uroporphyrin determination opens up possibilities for the further study of the relationship between porphyrin formation or excretion and clinical condition in this disease.

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